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Degree: Master of Science

Year this Degree Granted: 2003

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APOLIPOPROTEIN E - MEDIATED EFFECTS
ON NEURON GROWTH

by



Igor Cvetkovic

A thesis submitted to the Faculty of Graduate Studies and Research in
partial fulfillment of the requirements for the degree of

Master of Science

in

Experimental Medicine

Department of Medicine

Edmonton, Alberta

Fall 2003

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled “Apolipoprotein E - mediated effects on neuron growth” submitted by Igor Cvetkovic in partial fulfillment of the requirements for the degree of Master of Science in Experimental Medicine.

I dedicate this work to my parents, for shining their guiding light on my path to success, through turbulent times, from the war-torn former Yugoslavia to our new life in Canada, for their hard work, sound advice and understanding in times of need, and to my brother, for making sure his footprints of success did not fade in time until my turn had come to follow his lead and journey down the road "less travelled by".

You have made all the difference.

Abstract

Apolipoproteins are secreted proteins involved in transport and distribution of lipid molecules such as phospholipids, triacylglycerols and cholesterol. Recent studies have shown that human apolipoprotein E (**apoE**) also affects the growth of neuronal cell cultures. There are three major isoforms of apoE, namely apoE2, apoE3, and apoE4. Individuals who express the allele for apoE4 are predisposed to developing Alzheimer's disease. Several studies have suggested that the predominant apoE3 isoform stimulates neuronal growth, whereas apoE4 inhibits growth. The goal of this study is to examine whether human apoE3 and apoE4 exhibit different effects on axonal extension of rat sympathetic neurons.

Our results suggest that minimally lipidated human apoE3 and apoE4 exhibit no significant effects on axonal extension of rat sympathetic neurons grown in compartmented dishes. Even under conditions of reduced cholesterol biosynthesis in neurons, which promotes the binding and uptake of apoE particles, neither apoE3 nor apoE4 had any pronounced inhibitory effects on axonal extension.

Acknowledgements

This thesis would not be complete without acknowledging the efforts of those extraordinary people involved in the supervision, collaboration and technical support of this project. First and foremost, I would like to thank my supervisor Dr. Jean Vance for her patience, guidance, and support over the years, for allowing me to learn the intricate art of science, and for stimulating my interest in research. I would also like to thank the members of my supervisory committee, Dr. Robert Campenot, for sharing his vast knowledge and practical expertise in neuroscience, and Dr. Gordon Francis, for his mentorship in the field of lipoprotein metabolism. I also extend special thanks to Dr. Richard Jones, the Department of Medicine Director of Graduate Education, for acting as the chair of the examining committee.

I would also like to thank the individuals involved in the collaborative effort, namely Dr. Robert Ryan for his generous gift of apoE-transfected *E. coli* cells, Dr. David Williams for supplying apoE-secreting neuroblastoma cells, Dr. Linda Curtis for providing monoclonal anti-apoE antibody and Dr. Sabina Rustaeus for conducting preliminary work involving truncated apoE fragments.

I would also like to thank the members of the Vance laboratory, Dr. Hideki Hayashi, for his friendship and mentorship that made me a better scientist, Dr. Paul Grandmaison, for thoughtful suggestions and company during the long hours. Similarly, I thank Dr. Barbara Karten, Dr. Rineke Steenbergen, Sherry Selby, and Agnes Kulinski for their sound advice and trustworthy friendships.

I extend my thanks to Russ Watts for his technical support and reliable help with complex preparations of neuronal cultures. I also thank Karen Lund for her help with neuronal culture preparations, Priscilla Gao for culture medium preparations, and Laura Hargreaves for help with genotyping the mice.

I extend my thanks to Dr. Dennis Vance for organizing the Molecular and Cell Biology of Lipids (MCBL) group, Dr. Richard Lehner and Dr. Luis Agellon for the use of their laboratory facilities, as well as all the trainees of the MCBL group.

I would also like to thank the University of Alberta Department of Medicine for their guidance and support that made this project viable, and the Canadian Institutes of Health Research (CIHR) for funding this study.

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LIST OF ABBREVIATIONS

Units

°C	degrees Celsius
Ci	Curie
dpm	disintegrations per minute
m	metre
g	acceleration due to gravity
g	gram
Da	Daltons
L	litre
M	molar or mole per litre
mol	mole
RPM	revolutions per minute
v/v	volume per volume

Prefixes

k- (kilo-)	10^3
c- (centi-)	10^{-2}
m- (milli-)	10^{-3}
μ- (micro-)	10^{-6}
n- (nano-)	10^{-9}
p- (pico-)	10^{-12}

Abbreviations

Aβ	β -amyloid
apoE	apolipoprotein E
APP	amyloid precursor protein
β-ME	β - mercaptoethanol
BCA	bicinchoninic acid
bp	base pairs
BSA	bovine serum albumin
cAMP	adenosine-3',5'-cyclic monophosphate
CNS	central nervous system
CREB	cAMP-response element binding protein
CSF	cerebrospinal fluid
CTRL	control
Dab-1	disabled-1 adapter protein
DMEM	Dulbecco's modified Eagle's medium
DMPC	dimyristoylphosphatidylcholine
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide
dRS	delipidated rat serum
<i>E. coli</i>	<i>Escherichia coli</i>
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
F-12	Ham's F-12 medium
FBS	fetal bovine serum
HDL	high density lipoprotein
HMG-CoA	3-hydroxy-3-methylglutaryl co-enzyme A
HRP	horseradish peroxidase

HSPG	heparan sulphate proteoglycans
IDL	intermediate density lipoprotein
IgG	immunoglobulin G (antibody)
J-115	rabbit anti-rat apoE polyclonal antibody
JNK	c-Jun N-terminal kinase
LCAT	lecithin:cholesterol acyltransferase
LDL	low density lipoprotein
LDLR	LDL receptor
LRP	LDL receptor-related protein
MAPK	mitogen activated protein kinase
MW	molecular weight
MWCO	molecular weight cut-off
Neuro-2a	Neuroblastoma-2a cell line
NGF	nerve growth factor
PBS	phosphate-buffered saline
PBS-T	PBS with 0.05% Tween-20
PCR	polymerase chain reaction
PI3K	phosphatidylinositol 3-kinase
PKA	protein kinase A
PKB	protein kinase B
PVDF	polyvinylidene difluoride membrane
RAP	receptor associated protein
RS	rat serum
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SREBP	sterol regulatory element binding proteins
TLC	thin layer chromatography
TMB	3,3',5,5'-tetramethylbenzidine
Tris	tris (hydroxymethyl) aminomethane
VLDL	very low density lipoprotein

Amino acids

A	Ala	alanine
C	Cys	cysteine
H	His	histidine
K	Lys	lysine
L	Leu	leucine
N	Asn	asparagine
P	Pro	proline
R	Arg	arginine
S	Ser	serine
V	Val	valine
X	any	undetermined or any amino acid
Y	Tyr	tyrosine

Chemical Formulas

AgNO₃	silver nitrate
CH₃COOH	acetic acid
HCl	hydrochloric acid
KBr	potassium bromide
KCl	potassium chloride
KH₂PO₄	potassium dihydrogen phosphate
NaCl	sodium chloride
Na₂CO₃	sodium carbonate
NaHCO₃	sodium bicarbonate
NaH₂PO₄	sodium dihydrogen phosphate
Na₂HPO₄	disodium hydrogen phosphate
NaN₃	sodium azide
NaSCN	sodium thiocyanate
Na₂S₂O₃	sodium thiosulfate

CHAPTER I

Introduction

1.1 ALZHEIMER'S DISEASE

1.1.1 Introduction

In 1906, a psychiatrist by the name of Alois Alzheimer was first to describe a disorder that exhibited symptoms such as an impairment of cognitive function, progressive memory deterioration, loss of acquired intellectual and language skills, and adverse behaviour. Nearly one hundred years later, with the aging population on the rise and with prolonged life expectancies allowing individuals to reach ages where neurodegenerative conditions become frequent, conditions such as the one described by Alzheimer have risen to astounding levels in the elderly (Selkoe 2001a).

Alzheimer's disease is a debilitating progressive neurodegenerative disorder and is the major cause of dementia in the aged population (Gonzales McNeal et al. 2001). Approximately 50%-70% of dementia cases in the elderly can be attributed to Alzheimer's disease (Petrella et al. 2003). At early stages, the symptoms may include errors in judgment, forgetfulness, confusion, and impaired storing of new memories. Later stages of the disease are characterized by motor and sensory deficiencies. With the loss of coordination and impairment of gait, the symptoms often resemble those of the extrapyramidal motor disorders, such as Parkinson's disease (Selkoe 2001a). Brain atrophy accompanied with severe cholinergic deficiency underlies the detrimental effects caused by Alzheimer's disease. However, unlike Parkinson's disease, where predominantly dopaminergic neurons are affected, the degeneration in Alzheimer's disease affects multiple classes of neurons, making treatment very difficult. Computerized Tomographic and Magnetic Resonance Imaging scans show thinning of the cortical gyri, enlargement of the brain

ventricles, and atrophy of neurons in the areas of entorhinal cortex, hippocampus, and amygdala.

1.1.2 Characteristics of Alzheimer's disease

Aside from cortical atrophy and ventricular dilation in areas associated with memory, language and judgement, Alzheimer's disease is also characterized by formation of β -amyloid deposits, neurofibrillary tangles and cerebral amyloid angiopathy (Selkoe 2001a).

Senile plaques composed of fibrillar assemblies of β -amyloid peptides are derived from fragments of **amyloid precursor protein (APP)** (Selkoe 2001b). The normal function of APP is not yet clear. Virtually all cell types produce APP, and circulating β -amyloid peptides can also be detected in plasma, however APP is most abundantly expressed in neurons and other brain cells including astrocytes, microglia, endothelial and smooth muscle cells (Selkoe 2001a). Improper processing and proteolytic cleavage of APP by enzymes called secretases may result in the release of pathogenic β -amyloid fragments $A\beta_{40}$ and $A\beta_{42}$, which accumulate extracellularly in brain areas such as neocortex, hippocampus, basal ganglia, thalamus, and cerebellum. These fragments can aggregate to form β -amyloid plaques, which are characteristic of Alzheimer's disease.

Neurofibrillary tangles develop on the intracellular side of the plasma membrane of neurons. These fibrous bundles, characterised as paired helical filaments, are composed of aggregates of hyperphosphorylated microtubule-associated protein **tau**. The appearance of these tangles has been reported in other diseases as well, and they might form independently from β -amyloid plaques (Selkoe 2001a).

Cerebral amyloid angiopathy is present in the majority of Alzheimer's disease patients. It is characterized by the appearance of amyloid fibrils in the linings of the blood vessels of the brain and it can occasionally cause cerebral haemorrhage as amyloid-laden blood vessels rupture.

Two types of Alzheimer's disease have been described, an **early-onset** (familial) Alzheimer's disease which occurs in an autosomal dominant type manner, and **late-onset** Alzheimer's disease, which is associated with old age dementia (Selkoe 2001b). Both types seem to have identical symptoms. Early-onset Alzheimer's disease may strike before the age of 40 and can be attributed to mutations in amyloid precursor protein and presenilin genes. The majority (~90%) of Alzheimer's disease cases are classified as late-onset, and have been linked to memory decline in the elderly. The incidence of late-onset Alzheimer's disease has been correlated with the occurrence of the apolipoprotein E4 allele, and this phenomenon is the main topic of this study.

1.1.3 Alzheimer's disease and apolipoprotein E

A recent gene-dosage study has shown that 20% of individuals with no apoE4 alleles, 47% of individuals with one apoE4 allele, and 90% of individuals carrying two apoE4 alleles develop late-onset Alzheimer's disease (Corder et al. 1993). The mean age of onset for non-apoE4 individuals is 84.3, for heterozygous apoE4 is 75.5, and for homozygous individuals is 68.8 years of age. These studies suggest that apoE4 predisposes individuals to Alzheimer's disease and lowers the age of onset.

1.2 APOLIPOPROTEIN E

1.2.1 The structure of apoE

Apolipoprotein E (apoE) was first isolated in 1973 and was described as an arginine-rich protein (Shore and Shore 1973). ApoE is a 34 kDa single chain glycoprotein containing 299 amino acids. It contains two structural domains. An N-terminal 22 kDa domain consists of residues 1-191 and the C-terminal 10 kDa domain consists of residues 222-299 (Saito et al. 2003). The two domains are linked by a hinge-like region whose amino acid residues form a random structure. The N-terminal domain consists of a bundle of four amphipathic α -helices. Residues 134-150 located within helix 4 are responsible for binding to the **low density lipoprotein (LDL) receptor**, and several basic amino acid lysine residues have been implicated in this function (Saito et al. 2003). The C-terminal domain also contains α -helical structures and is responsible for binding to hydrophobic lipid regions of lipoprotein particles (**Figure1.1**).

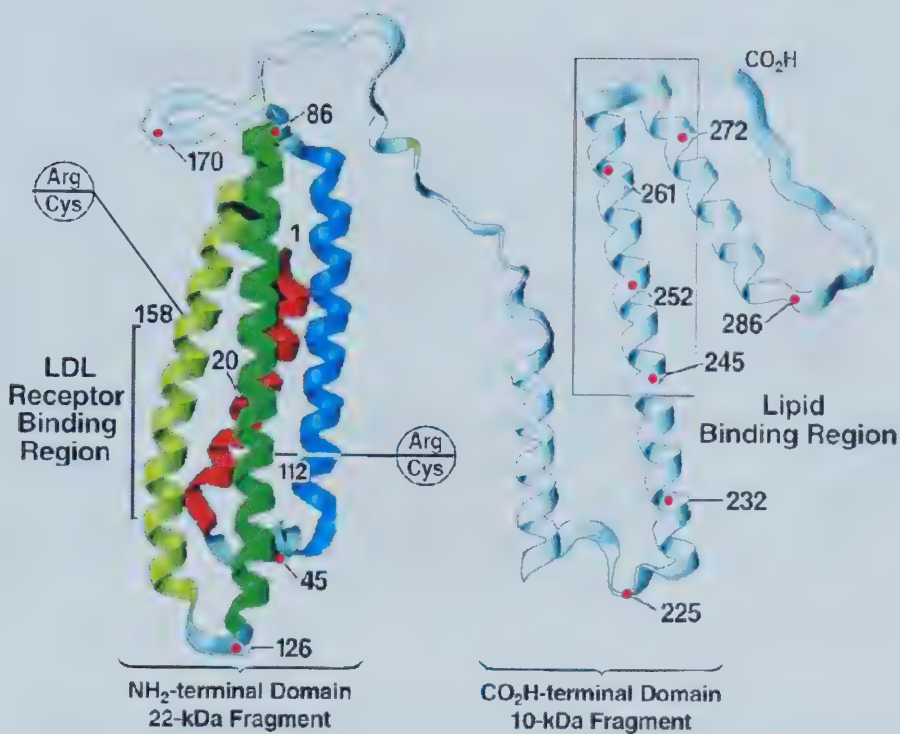


Figure 1.1 : Apolipoprotein E structure. The N-terminal 22 kDa domain contains the LDL receptor-binding domain on helix 4, while the C-terminal contains the lipid-binding domain. Figure adapted from (Huang et al. 2001)

ApoE can be glycosylated with N-acetylneuraminic acid (sialic acid) residues, which impart a negative charge. Numerous salt bridges are located in the four-helix bundle, and serve to stabilize the structure of the folded protein. **Figure 1.2** shows a proposed mechanism of apoE interaction with lipids. Each amphipathic helical structure contains a hydrophobic face which associates with the lipids in the monolayer of lipoprotein particles, and a hydrophilic face which is exposed to plasma. This amphipathic property permits the lipoprotein particle to remain soluble in the bloodstream, and allows for a transport of hydrophobic molecules in the aqueous environment.

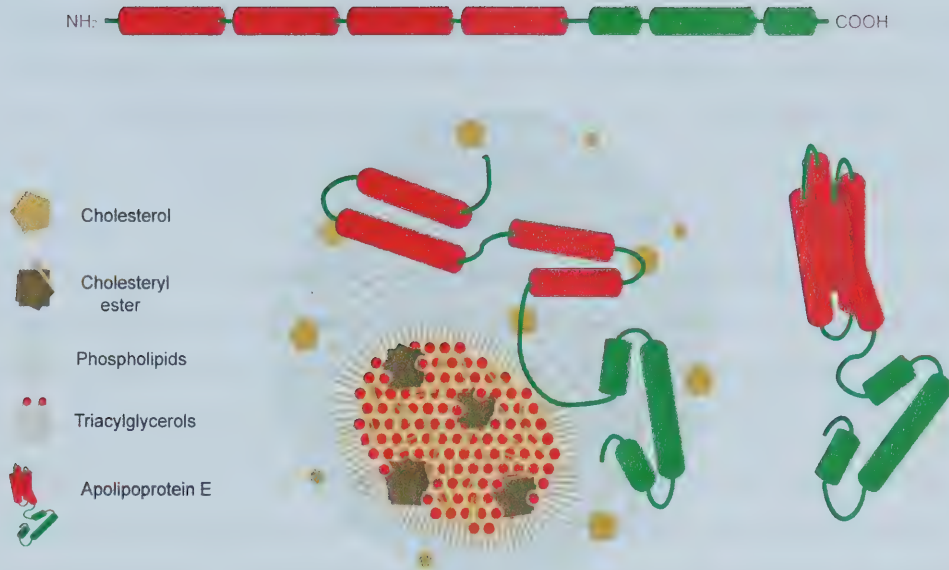


Figure 1.2 : Apolipoprotein E binds to lipoprotein particles.

The secondary structure of apoE (top) has numerous α -helical domains (*cylinders*). The N-terminal domain of apoE (*red*) has a proposed open conformation when bound to a lipoprotein particle (*center*), and a closed conformation when lipid-free (*right*). This hinged domain model is based on apoE interaction with lipids proposed by Narayanaswami (Narayanaswami and Ryan 2000). The lipid particle also contains other apolipoproteins.

1.2.2 The origin of apoE production

ApoE is primarily involved in plasma lipid transport but has also been found to affect nerve growth and play an active role in both healthy and diseased brain tissue. Many organs synthesize apoE, including the liver, brain, lung, kidney, spleen and adrenal glands (Lin et al. 1986). The liver is the major site of apoE production. Hepatic parenchymal cells are responsible for production of 75% of the circulating plasma apoE (Laws et al. 2003). The brain is the second largest producer of apoE. In the **central nervous system (CNS)**, astrocytes are the main apoE-secreting cells, and

since cerebrospinal fluid lacks apoB, apoE is thought to be the main cholesterol and lipid transport protein. Central nervous system and peripheral pools of apoE are thought to be separated by the blood-brain-barrier. Most studies have reported normal apoE concentrations to be in the range of 5 µg/ml in cerebrospinal fluid and up to 100 µg/ml in the serum (Carlsson et al. 1991). In patients with acute and chronic neuro-inflammatory and cardiovascular diseases, apoE concentrations in the cerebrospinal fluid can easily double. Local concentrations of apoE at the site of spinal cord injury or head trauma can be much higher. Recent studies have shown a surge of apoE mRNA expression and apoE protein levels in mice whose spinal cords have been transected, as early as 4 days after injury, and remain up-regulated beyond 3 weeks (Seitz et al. 2003). The neutrophils and macrophages associated with the inflammatory response after spinal cord injury are thought to be the main source of apoE. This suggests involvement of apoE in recycling of lipids at the site of damage and its role during nerve remodelling. Similarly, after peripheral nerve injury, apoE is thought to be involved in the recycling of the cellular debris, as distal axons and Schwann cells distal to the site of injury degenerate (**Wallerian degeneration**) (Goodrum 1991). Blood-derived macrophages are responsible for the re-uptake of the cellular debris, lipids and cholesterol. The subsequent secretion of apoE cholesterol-rich lipoproteins re-supplies the regenerating neurons and Schwann cells with lipids and cholesterol necessary for their growth and regeneration. However, it has also been reported that this regeneration process is not entirely dependent on apoE, since apoE deficient mice regenerate neurons at a normal rate (Goodrum et al. 1995).

1.2.3 ApoE isoform polymorphism

ApoE gene is located on chromosome 19 (locus 19q13.2), and it is composed of four exons and three introns. Three major isoforms of human apoE are determined by a polymorphism of alleles at a single gene locus. These isoforms of apoE are determined by cysteine-arginine amino acid substitutions at positions 112 and 158. **ApoE3** is the most common isoform and has a cysteine amino acid residue at position 112 and an arginine residue at position 158. **ApoE2** has cysteine residues at both locations, while **apoE4** has arginine residues at both positions. The North American frequency distribution of alleles is approximately 0.77 for apoE3, 0.15 for apoE4, and 0.08 for apoE2. The distribution of the six genotypes is approximately 55% E3/3, 25% E3/4, 15% E3/2, and the remaining 5% are E4/4, E4/2, or E2/2 (Mahley and Rall 2000).

ApoE3 is considered to be normal and by far the most common allele. In comparison to apoE3, it is interesting to note that a single cysteine to arginine amino acid substitution in apoE4 predisposes individuals to development of Alzheimer's disease. Since arginine is a positively charged amino acid, charge distribution may play an important part in determining the functional properties of this protein, as well as the overall conformation of each isoform (Lund-Katz et al. 2001). Other pathogenic mutant isoforms (E1, E5-E7) also exist, however they are very rare.

1.2.4 The function of apolipoproteins

As a secretory protein, apoE is involved in the transport of cholesterol, triacylglycerols, and phospholipids. ApoE is an exchangeable apolipoprotein that is found on plasma lipoprotein particles, such as **chylomicrons, very low density lipoproteins (VLDL), intermediate**

density lipoproteins (IDL) and **high density lipoproteins (HDL)**. Its role is to stabilize the lipoprotein and to serve as a ligand for lipoprotein receptors.

Other apolipoproteins such as the **apoAs**, **apoBs**, and **apoCs** are also involved in the transport and metabolism of lipids (Davis and Vance 1996). ApoA1 is involved in the HDL-mediated reverse cholesterol transport that delivers lipids from the periphery back to the liver. These lipoproteins are then removed from plasma via the apoE receptor-mediated pathway involving the LDL receptor-related protein or the **scavenger receptor (SR-B1)** pathway. The HDL protein fraction is primarily composed of exchangeable apolipoproteins, with apoA1 being the major one (Fielding and Fielding 2002). Similar to apoE, apoA1 is also found in CNS. However, unlike brain-derived apoE, which is locally produced by glial cells, apoA1 is synthesized in the liver and can reach the brain by crossing the blood-brain barrier (Koch et al. 2001). ApoA1 stimulates cholesterol efflux from cells and activates **lecithin:cholesterol acyltransferase (LCAT)** enzyme which can esterify cholesterol and transfer acyl groups among phospholipids. ApoA2 on the other hand has inhibitory effects on apoA1-mediated LCAT activation. ApoB does not exchange among lipoproteins, and is found on the surface of chylomicrons, VLDL, IDL, and LDL particles that deliver lipids to peripheral tissues. The outer layer of these particles may also contain exchangeable apolipoproteins apoE and apoCs embedded in a monolayer consisting of phospholipids and small amounts of cholesterol, while the core of these particles contains hydrophobic triacylglycerols and cholesteryl esters. Both apoB and apoE can bind to the LDL receptor. However, unlike apoE, which is locally produced in the brain, apoB is not found in CNS. ApoC2 activates lipoprotein lipase, which hydrolyzes lipoprotein triacylglycerols to release unesterified fatty acids at the cell surface, while apoC3 inhibits apoC2

activity. Similar to apoE, other apolipoproteins such as the **apoD** and **apoJ** have also been implicated in the CNS lipid homeostasis.

1.3 APOLIPOPROTEIN E RECEPTORS

ApoE on lipoprotein particles interacts with the LDL family of receptors that are found on cell plasma membranes. Positively charged arginine amino acid residues located in the N-terminal region 134-150 of apoE (RVRLASHLRKLRKLLR) are critical for binding to the LDL receptors (Hussain et al. 1999). The interaction between the two moieties is responsible for internalization of lipoproteins into clathrin-coated vesicles that are endocytosed into the cell (Brown et al. 1997). Endocytic pathways deliver receptor-ligand complex to acidic endosomes where the ligands are released. The ligands are then hydrolyzed in lysosomes, while the receptors are recycled back to the cell surface.

1.3.1 **LDL receptor family**

ApoE can bind numerous receptors of the LDL receptor family, such as **LDL receptor (LDLR)**, **VLDL receptor (VLDLR)**, **LDLR-related protein (LRP)**, **megalin**, **apoE receptor 2 (ER2)**, and **LR11**. These receptors are trans-membrane glycoproteins that share many structural and functional properties. **Figure 1.3** shows a general structural model of the LDL receptor. The receptors are expressed on the cell surface, and have an extracellular ligand-binding domain composed of a cluster of cysteine-rich repeats, stabilized by disulfide bridges. **Epidermal growth factor (EGF)** precursor homology domains are necessary for dissociation of the ligand from the receptor in endosomes. The function of the O-linked sugar domain is not yet clear, but it might serve to stabilize the receptor. A hydrophobic

single membrane spanning region connects to a C-terminal region of the receptor. A highly conserved cytoplasmic domain contains an internalization signal sequence Asn-Pro-x-Tyr (**NPxY**), which targets receptors to clathrin-coated pits for internalization via receptor-mediated endocytosis. The cytoplasmic domain also interacts with various adapter and scaffold proteins involved in cell signalling. NPxY domain usually interacts with phosphotyrosine binding domains of adapter proteins (Herz and Bock 2002).

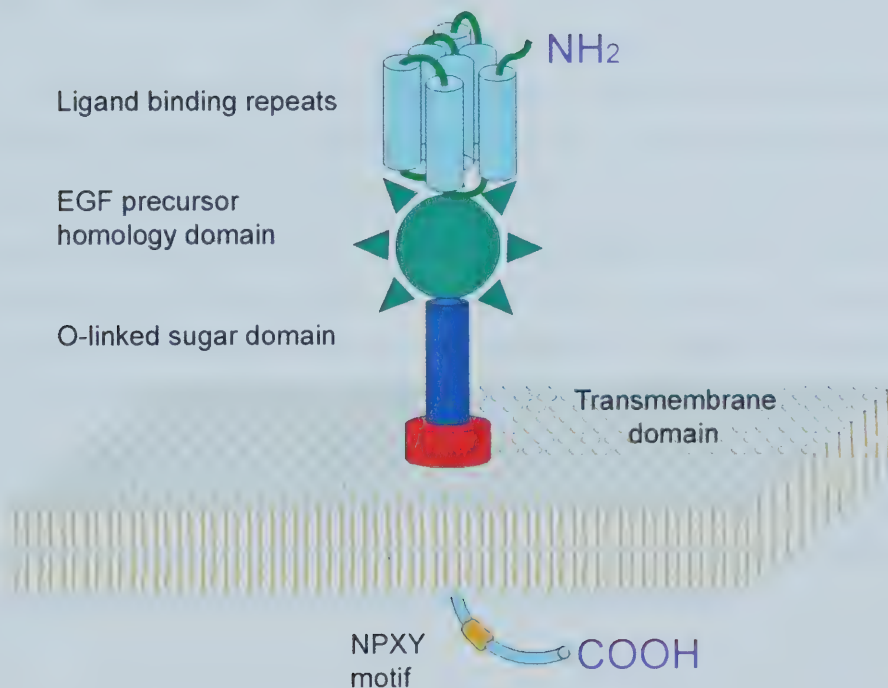


Figure 1.3 : Structure of the LDL receptor. The ligand-binding domain consists of seven repeats of ~40 amino acids each. Each repeat has clusters of negatively charged residues and six cysteine residues which form disulfide bridges. ApoE ligand is positively charged with arginine residues and its binding is dependent on residues in the repeat 5 of the LDLR ligand-binding domain (Brown et al. 1997).

Members of the LDL receptor family share high degrees of sequence homology and this is evident over a wide range of species. This homology was achieved by duplication and exon shuffling originating from the ancestral gene (Schneider et al. 1997). Differential splicing of the gene resulted in different ligand binding specificities. Many apoE-related studies have focused their attention on two receptors in particular, the LDL receptor and the LRP receptor.

1.3.2 LDL receptor (LDLR)

The pioneering work of Brown and Goldstein in the 1970s has resulted in the identification of the LDL receptor and characterization of the receptor-mediated cholesterol metabolism (Brown and Goldstein 1986). The LDL receptor is involved in cholesterol homeostasis and its expression is regulated by growth factors, hormones and unlike other LDLR family members, by intracellular sterol levels (Hussain et al. 1999). It is proposed that under conditions of low sterol levels, the transcription factors such as **sterol regulatory element binding proteins (SREBP)** are cleaved in the endoplasmic reticulum, make their way to the nucleus and bind to **sterol regulatory elements (SRE)** in the LDLR promoter region to enhance the transcription of the LDLR gene (Brown and Goldstein 1997).

Mutations in the LDLR result in **familial hypercholesterolemia**, an autosomal dominant disease characterized by increased plasma cholesterol levels and premature development of coronary heart disease. Both apoE3 and apoE4 bind to the LDL receptor with the similar affinity, while apoE2 has a much lower affinity (Lund-Katz et al. 2001). Furthermore, apoE-containing lipoproteins bind with a much higher affinity to the LDLR, in comparison to apoB-only lipoproteins (Mahley and Rall 2000).

1.3.3 LDL receptor-related protein (LRP)

The LRP receptor is also involved in clearance of apoE. Both LDLR and LRP are found in the liver, but in comparison to LDLR, which is found on virtually all cell types, LRP is most abundant in liver and brain (Herz and Bock 2002). While LRP is not likely to bind apoB, it binds to apoE and other ligands such as **receptor-associated protein (RAP)** (Hussain et al. 1999). LRP is also proposed to bind amyloid precursor protein. Recent studies have suggested that LRP is involved in clearance of soluble β -amyloid pathogenic proteins $A\beta_{42}$ and $A\beta_{40}$ via receptor-mediated uptake, and that reduced levels of LRP are correlated with an increased risk of Alzheimer's disease (Kang et al. 2000). These studies suggest that apoE4 impedes LRP clearance mechanisms of β -amyloid and promotes fibrillogenesis. It is suggested that apoE2 and apoE3 isoforms promote neuronal survival by enhanced clearance of these soluble $a\beta$ complexes.

However, it remains unclear whether the LRP receptor is mainly responsible for the differential effects of apoE3 and apoE4. It was recently reported that apoE4 by itself is capable of inducing neurotoxicity without the involvement of LRP (Hagiwara et al. 2000).

1.3.4 The role of heparan sulfate proteoglycans (HSPG)

Numerous studies have suggested that apoE binding to the receptors is dependent on cell surface **heparan sulfate proteoglycans (HSPG)** (Mahley 1996). It was shown that addition of apoE to β -VLDL enhances the uptake of these particles, and treatment with the enzyme heparinase, which disables the function of heparin proteoglycans, reduces the enhanced uptake of apoE-rich β -VLDL particles in HepG2 (hepatoma)

and **Chinese hamster ovary (CHO)** cells (Ji et al. 1993). This mode of interaction is not just limited to apoE-containing β -VLDL. Binding of HDL particles in an apoE-mediated manner is also thought to be dependent on HSPG (Ji et al. 1997). Thus, it has been suggested that the interaction of apoE with cell-surface HSPG is an initial step in the clearance of lipoproteins from the plasma, after which, the apoE-containing lipoproteins bind to the LRP receptor and are internalized by the cell.

Both the N-terminal and the C-terminal domains of apoE contain a heparin binding site (Saito et al. 2003). The N-terminal domain site, which is thought to be the predominant HSPG binding site, is located between apoE residues 142-147 and overlaps with the LDL receptor binding region.

1.4 APOLIPOPROTEIN E RECEPTORS AND INTRACELLULAR SIGNALLING

Aside from being a lipid transport protein, apoE has been implicated in the receptor-dependent signal transduction and intracellular signalling pathways. A recent study has suggested that in rat hippocampal neurons, the binding of apoE4 or neurotoxic apoE peptides, but not apoE3, results in Ca^{2+} influx and the **cAMP-dependent protein kinase (PKA) / extracellular signal regulated kinase (ERK)** dependent phosphorylation cascade that activates **cAMP-response element binding proteins (CREB)** (Ohkubo et al. 2001). These CREB proteins activate the transcription of many genes such as c-fos and Bcl-2, which play a role in neuronal survival and plasticity (over-expression of Bcl-2 can inhibit neuronal death). Other studies have shown that members of the LDLR gene family are not only involved in the endocytosis of their ligands, but also interact with many cytoplasmic adaptor proteins (Gotthardt et al. 2000). These adapter proteins may activate

intracellular kinases which may affect cytoskeletal remodelling, cell adhesion and neuronal migration (Herz and Bock 2002). Furthermore, a secreted protein **Reelin**, which is important in neuronal migration and brain development, has been shown to interact with VLDLR and apoER2 receptors, which in turn may cluster with other proteins capable of activating tyrosine kinases that phosphorylate adapter proteins such as **Disabled-1 (Dab1)** and transmit the signal to kinases which can phosphorylate cytoskeletal proteins such as microtubule-associated protein tau.

1.5 APOLIPOPROTEIN E DEFICIENCY

ApoE deficiency has been shown to promote atherosclerosis (Zhang et al. 1992). ApoE plays a critical role in the clearance of lipoproteins and apoE deficient mice have high levels of plasma cholesterol, develop atherosclerotic lesions in the blood vessels, and have a reduced life span (Moghadasian et al. 2001). Similarly, the inhibition of production of apoE mRNA in mice by administration of apoE anti-sense oligodeoxynucleotides increases their plasma cholesterol and triacylglycerol levels (Morishita et al. 2002). Other studies have shown that administering apoE to apoE-deficient mice significantly lowers their high plasma cholesterol levels and protects against atherosclerosis (Tsukamoto et al. 1999). The apoE3 isoform seems to have the best protection against atherosclerosis; however the reasons for this are yet unclear.

ApoE deficiency also impairs cognitive function (Raber et al. 1998). Raber et al. have conducted behavioural studies with apoE deficient mice and found that their central nervous system developed normally, however at six months of age the mice exhibited neurodegenerative impairments. Their

nerve regenerative capabilities after brain lesions were reduced and learning deficits were observed.

1.6 APOE ISOFORM-SPECIFIC EFFECTS ON NERVE GROWTH

In order to analyze the function of apoE in nerve growth and degeneration, a closer look must be taken at apoE isoform-specific effects on nerve growth. Human apoE3-transgenic mice lacking endogenous mouse apoE behave similarly to wild-type mice, however apoE4-transgenic mice and apoE-deficient mice showed impairments in learning and exploratory behaviour (Raber et al. 1998). These impairments increased with age. Another study has shown that possession of apoE4 allele may be associated with increased severity of chronic neurological deficits caused by head traumas in high-exposure boxers (Jordan et al. 1997). As suggested by the correlation between Alzheimer's disease and apoE4 allele, these findings support the growing evidence that apoE has isoform-specific effects on brain function. Numerous studies have been performed in order to account for the differences between the apoE3 and apoE4 isoforms. While apoE3 was generally found to stimulate the growth of neuronal cell lines, the effect of apoE4 was rather controversial, and has been reported as inhibitory (Nathan et al. 2002), neutral (Buttini et al. 1999), or stimulatory (Puttfarcken et al. 1997).

1.6.1 ApoE fragments have neurotoxic properties.

Some studies have indicated that apoE-derived peptide residues 141-155 are neurotoxic to superior cervical ganglion cells of rats, and hippocampal neurons of mice (Moulder et al. 1999). Another study shows

that thrombin-cleaved apoE3 and apoE4 N-terminal fragments are both neurotoxic (Marques et al. 1996). This toxicity was shown to be dependent on the heparan sulfate proteoglycan-linked LRP receptor mediated pathway (Tolar et al. 1997). However one must realize that these findings are limited by the fact that these are just fragments and not physiological forms of apoE.

1.6.2 ApoE3 stimulates, while apoE4 inhibits, axonal growth.

It has been shown that the addition of β -VLDL particles enriched with apoE3 increased neurite outgrowth from cultured Neuro-2a cells, while the addition of apoE4 inhibited the outgrowth (Nathan et al. 1995). This difference in growth was attributed to the observation that apoE4 isoform might be involved in de-polymerization of cytoskeletal microtubules. This was further supported by evidence that apoE4-treated cells contained fewer microtubules and more monomeric tubulin than apoE3-treated cells. A follow up study using cortical neurons from adult mice confirmed an inhibitory effect of human apoE4 on neurite outgrowth, and demonstrated that these isoform-specific effects were dependent on the LRP receptor (Nathan et al. 2002).

Another study has shown that mouse neuroblastoma cells transfected with human apoE3 and apoE4 had similar neurite outgrowths, and only in the presence of exogenously administered lipids did apoE4 show inhibitory effects (Bellosta et al. 1995). However, the physiological relevance of this study is limited by the fact that neurons do not normally make apoE. Other research has shown that outgrowth and sprouting of hippocampal slice cultures isolated from human-apoE4 transgenic mice were only 50% that of mice transfected with human apoE3 (Teter et al.

2002). The overall sprouting density was examined using a sprouting index obtained by measuring the optical density of the neurites stained by Timm's stain followed by the quantitative image analysis. The effects were dependent on apoE4 transgene copy number, where two transgene copies of apoE4 caused larger defects in sprouting than a single transgene. This gene-dosage effect has suggested a neurotoxic property of apoE4.

1.6.3 ApoE3 stimulates axonal growth, while apoE4 is neutral.

Expression of human apoE3 in apoE ^{-/-} mice has a protective effect against excitotoxic oxidative stress and age-dependent neurodegeneration, comparable to that observed in non-transfected wild-type mice (Buttini et al. 1999). ApoE3 prevented age-dependent neurodegeneration of pre-synaptic terminals and dendrites, as well as axons in the hippocampus of these transgenic mice, whereas apoE4 did not. ApoE4 transgenic mice were as unprotected against neurodegeneration as apoE ^{-/-} mice. Although apoE4 did not protect against neurodegeneration in mice, no additional inhibitory effect by apoE4 was observed in this study.

1.6.4 Both apoE3 and apoE4 stimulate axonal growth.

A recent study has shown that both apoE3 and apoE4 have neurotrophic effects on neuron growth (Puttfarcken et al. 1997). In this study, lipid-associated human apoE3 and apoE4 particles were applied to developing cultures of rat hippocampal neurons and the lengths of their neurites were measured. Both isoforms exhibited neurotrophic effects, as indicated by increased neurite lengths. Similarly, both apoE3 and apoE4 isoforms reduced the neurotoxicity of A β ₄₂ fragments.

1.7 β -AMYLOID CLEARANCE

β -amyloid plaque deposit density is correlated with apoE4 genotype. An analysis of the interaction of apoE2, apoE3, and apoE4 with A β ₄₀ fragments *in vitro* showed that apoE2 binds to β -amyloid with the highest affinity, and that both apoE2 and apoE3 bind much more efficiently than apoE4 (Aleshkov et al. 1997). Purified delipidated forms of these proteins showed little isoform specificity, indicating that the level of apoE lipidation influences its conformation and its biological activity (LaDu et al. 1995). In fact, in comparison to apoE4, physiological forms of apoE3 isolated from plasma bind with a 20-fold greater affinity to β -amyloid. Hence, the correlation between apoE4 allele and high β -amyloid levels in plasma of Alzheimer's disease patients might be due to the inability of this apoE isoform to remove soluble forms of β -amyloid (Kang et al. 2000).

Recent studies have examined the effects of apoE on β -amyloid-induced toxicity in rat hippocampal neurons. It was found that β -amyloid protein is neurotoxic to cells cultured *in vitro*, and that apoE3, unlike apoE4, forms a SDS-stable complex with β -amyloid (Jordan et al. 1998). They hypothesized that apoE3 removes β -amyloid by clearing it via apoE receptors and that neurotoxicity is thereby reduced in this receptor-mediated process. This hypothesis was supported by the fact that apoE3 treatment had a protective effect against β -amyloid. Most importantly, the fact that the LRP receptor inhibitor, receptor associated protein (RAP), eliminated apoE3 protection against β -amyloid formation suggests that the LRP receptor is involved in removal of β -amyloid in complex with apoE3.

1.8 CHOLESTEROL IN BODY AND BRAIN

The brain as an organ plays an active role in the transport and metabolism of lipids, however, the blood-brain-barrier shields the brain from systemic lipoproteins. The brain has its own biosynthetic machinery for most of its cholesterol production, as well as its own independent supply of apolipoproteins. It is reported that more than one half of the brain's mass is composed of lipids, and it contains 25% of the body's unesterified cholesterol (Herz and Bock 2002).

1.8.1 The importance of cholesterol in Alzheimer's disease

In comparison to apoE3, individuals carrying the apoE4 allele tend to have increased plasma cholesterol levels. A recent review by Yanagisawa has emphasized the implications of high cholesterol levels in the pathogenesis of Alzheimer's disease (Yanagisawa 2002). It has also been suggested that reducing intracellular cholesterol levels decreases the cleavage of amyloid precursor protein (APP) and reduces the production of β -amyloid peptides (Simons et al. 1998). The APP processing machinery was found to be associated with detergent-insoluble domains of plasma membranes called **lipid rafts** (Ehehalt et al. 2003). These cholesterol and sphingolipid-rich microdomains of the plasma membrane are highly ordered and tightly packed, in comparison to the normal lipid composition of the plasma membrane, and can diffuse laterally within the membrane. The rafts are involved in membrane trafficking of proteins, cholesterol transport and cellular signaling (Simons and Ehehalt 2002). Recent studies have demonstrated that lipid rafts contain APP, presenilins, and secretases involved in production of β -amyloid, and may play an important role in the development of Alzheimer's disease (Riddell et al. 2001). Cholesterol was shown to be important in the maintenance of functional lipid rafts, as its

hydrophobic properties allow for the proper raft assembly. Under reduced cholesterol conditions APP biosynthesis is not affected, however β -amyloid production is reduced (Simons et al. 1998). This might be due to a reduced transport of APP to lipid rafts, or by reduced activity of secretases under low cholesterol environment.

1.8.2 Cholesterol biosynthesis

Cellular cholesterol levels are regulated by many mechanisms which include the regulation of LDL receptor expression, rate of esterification to cholesteryl ester, and cholesterol *de novo* synthesis (**Figure 1.4**).

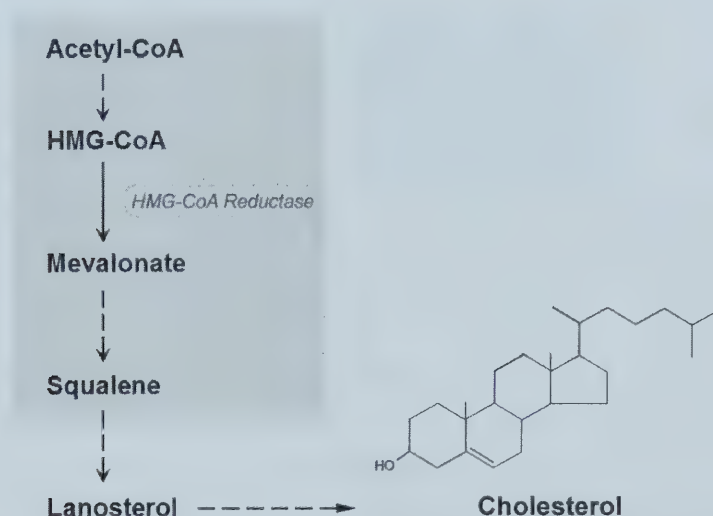
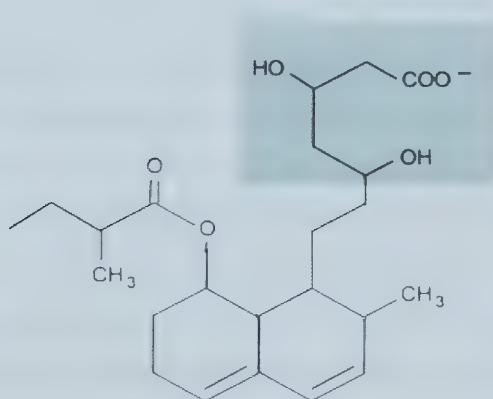


Figure 1.4 : Cholesterol biosynthesis pathway. Cholesterol is synthesized from acetyl-CoA through a series of reactions. HMG-CoA reductase is the key regulatory enzyme in this pathway. The shaded area indicates all the open-chain precursors prior to their cyclization into a tetracyclic steroid skeleton of lanosterol. Lanosterol is further processed into a final cholesterol molecule (Voet and Voet 1995).

3-hydroxy-3-methylglutaryl co-enzyme A (HMG-CoA) reductase is the rate-limiting enzyme of cholesterol biosynthesis, and it reduces HMG-CoA to mevalonate. This enzyme is regulated by competitive and allosteric inhibition, phosphorylation, and feedback regulation by cholesterol. Many studies have used cholesterol synthesis inhibitors such as lovastatin, simvastatin, or pravastatin to study cellular metabolism under reduced cellular cholesterol levels. These competitive inhibitors of HMG-CoA reductase are not only able to reduce systemic serum plasma cholesterol levels (pravastatin is hydrophilic and thought not to cross the blood-brain-barrier), but also cholesterol levels in the brain (simvastatin and lovastatin are lipophilic and can penetrate the blood brain barrier) (Kirsch et al. 2003). An example of a statin, **compactin** (mevastatin), is shown in **Figure 1.5**.



Compactin

Figure 1.5 : Structure of compactin. The shaded area indicates a structure that resembles HMG-CoA and mevalonate. Compactin competitively inhibits HMG-CoA reductase, a key regulatory enzyme of cholesterol biosynthesis. The statins usually block the active site of the enzyme by occupying a portion of the binding site reserved for HMG-CoA (Istvan 2002). Figure adapted from Voet and Voet (Voet and Voet 1995).

Epidemiological studies have shown that patients treated with cholesterol synthesis inhibitors are at a reduced risk of developing dementia (Jick et al. 2000). These inhibitors have also been shown to reduce intracellular and extracellular levels of A β ₄₂ and A β ₄₀ peptides in primary cultures of hippocampal neurons (Fassbender et al. 2001). It was also demonstrated that re-addition of cholesterol to cholesterol-depleted cells can initiate β -amyloid production and secretion, and ultimately reverse the effects of cholesterol inhibitors (Simons et al. 1998).

It is currently unknown how a reduction in cholesterol synthesis affects the production of β -amyloid and development of Alzheimer's disease. A recent study has shown that synaptic plasma membranes of young mice show an asymmetric distribution of cholesterol with the exofacial leaflet having 15% and cytofacial leaflet 85% of total membrane cholesterol (Igbavboa et al. 1996). In older mice, the cholesterol in the exofacial leaflet can double to 30% of the total membrane cholesterol, while the total cholesterol content remains the same. An increase in exofacial leaflet cholesterol content was also evident in the apoE-deficient and LDL receptor-deficient mice (Igbavboa et al. 1997). Another study has shown that while total synaptic plasma membrane cholesterol levels were similar in apoE3- and apoE4- transgenic mice, the distribution of cholesterol between the inner and outer leaflets of the plasma membrane differed (Hayashi et al. 2002). In comparison to wild-type and apoE3 transgenic mice, the cholesterol levels in the apoE4 transgenic mice were increased on the exofacial leaflet and decreased in the cytofacial leaflet in the plasma membranes. Since apoE4 is correlated with Alzheimer's disease, these findings indicate a possible link between membrane cholesterol distribution and development of β -amyloid plaques found in the Alzheimer's disease. The exact mechanisms of this interaction are yet to be determined.

The distribution of cholesterol in the plasma membrane may be important for the proper function of membrane components. The amphipathic β -amyloid fragments tend to interact with lipids, however the role of β -amyloid in lipid metabolism is poorly understood. Composition changes in lipid rafts may alter their function, increase the processing of APP, or increase the binding of β -amyloid to cell surface. It was recently shown that β -amyloid increased membrane fluidity in cerebral cortex and hippocampus in rats (Chochina et al. 2001). It is suggested that the change in the cholesterol membrane distribution might enhance the interaction of β -amyloid with cholesterol and provide a suitable environment for β -amyloid production or accumulation (Wood et al. 2002). Although these results suggest a strong link between cholesterol and Alzheimer's disease, further studies are necessary to elucidate the mechanisms of these interactions.

1.9 PRELIMINARY EXPERIMENTS

All these previous studies suggest that lipids play an important role in the development of Alzheimer's disease. Previous research in our laboratory has focused on lipid metabolism in primary neurons, specifically on the roles of cholesterol and lipoproteins during axonal growth and regeneration. One of our primary interests is to verify whether cholesterol is a determining factor in the rate of neuronal extension of axons. A recent review of apoE isoform-specific effects has suggested that lipid delivery might not be the primary reason for differences in effect of apoE isoforms on neurons (Fagan and Holtzman 2000). Many studies have utilized different types of lipids in the analysis of apoE isoform differences and have concluded that apoE3 is more beneficial than apoE4. These lipids however, ranged from cholesterol-free **dimyristoyl-phosphatidylcholine (DMPC)** phospholipids (Ji et al. 2002), to VLDL (Michikawa and Yanagisawa 1998)

and minimally lipidated lipoprotein particles (DeMattos et al. 2001). Hence, a specific type of lipid may not be the determining factor of apoE isoform differences. The delivery of lipids themselves might not be as important as their role in making apoE functional, since purified delipidated forms of apoE show no isoform-specific effects on neuronal cells. A recent study by Michikawa and Yanagisawa indicated that in rat cerebral cortex neurons, inhibition of cholesterol synthesis by compactin induces cell death in the presence of apoE4 and β -VLDL, but not in the presence of apoE3. The addition of cholesterol biosynthetic precursors mevalonate and squalene rescued these neurons (Michikawa and Yanagisawa 1998). These results on the other hand, suggest that the inability of apoE4 to deliver lipids might be the causal factor.

In our laboratory, preliminary experiments using N-terminal fragments of apoE associated with dimyristoyl-phosphatidylcholine (DMPC) lipid (as illustrated in **Figure 1.6**), suggested that the truncated N-terminal domain of apoE3 and apoE4 must be lipid-associated in order to be functional.

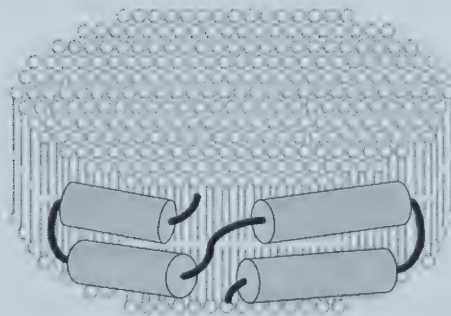


Figure 1.6 : Association of the N-terminal 22 kDa fragment of apoE with lipids. Amphipathic amino acid residues orient their hydrophobic side towards the hydrophobic regions of the phospholipids. Recent evidence suggests that hydrophobic regions of the four-helix bundle orient perpendicularly towards the fatty acyl chains of the phospholipids. Figure based on model suggested by Raussens et al. (Raussens et al. 1998).

It was also demonstrated that cholesterol synthesis inhibitors such as pravastatin and compactin inhibit axonal elongation when added to cell bodies/proximal axons of rat sympathetic neurons. Moreover, only in the presence of compactin did N-terminal domain of apoE4 show a pronounced inhibition of axonal extension in rat sympathetic neurons. However, unlike in the experiments presented by Michikawa and Yanagisawa, where cholesterol biosynthetic precursors rescued the cells treated with compactin and apoE4, we have found that the addition of the cholesterol precursor mevalonate, or cholesterol itself, did not reduce the inhibition of axonal extension imparted by compactin and apoE4. This has suggested that the apoE isoform-specific effect on axonal extension is not determined by the delivery of lipid or cholesterol, but by some other possible apoE-mediated signalling effect. This signalling effect is also suggested by other studies which show that minimally lipidated full-length apoE3 and apoE4 lipid particles with similar lipid and cholesterol content exhibit isoform-specific effects on nerve growth (DeMattos et al. 1998). This particular observation along with the role of apoE4 isoform during axonal extension is, therefore, the main focus of this study.

1.10 HYPOTHESIS

Based on previous studies, our hypothesis is that apoE4 inhibits axonal extension of primary rat sympathetic neurons by a mechanism independent of lipid uptake. We suggest that the apoE4 interacts with the LDL receptor and induces an inhibitory signal during axonal extension. We also predict that an inhibition of cholesterol synthesis in neurons will up-regulate the expression of the LDL receptor, which will increase neuronal

binding of apoE4, and in turn initiate an enhanced inhibition of axonal growth.

We will also establish a PCR-based system for genotyping LDLR $-/-$ mice to be used in future experiments. If, in fact, the LDLR is the key receptor involved in propagating the apoE4-induced inhibitory signal on axonal extension, in LDLR $-/-$ mice, all inhibitory effects of apoE4 will be eliminated. Since we use minimally lipidated apoE particles, whose isoform-specific effects may not be dependent on lipid delivery, the absence of the LDLR will allow us to examine whether this receptor is involved in an apoE4-induced inhibitory signalling.

1.10.1 Objectives

Our goal is to purify minimally lipidated apoE particles and apply them to rat sympathetic neurons in the presence or absence of cholesterol synthesis inhibition. We will attempt to determine whether apoE3 and apoE4 isoforms have different effects on axonal extension of these neurons. Several models of apoE are at our disposal, including the human apoE N-terminal fragments (1-183) secreted by transformed *E. coli* cells, and the full-length human apoE3 and apoE4 on lipidated particles secreted by cultured neuroblastoma cells.

Neurons will be grown in compartmented dishes that contain teflon dividers which permit cell bodies and axons to grow in separate compartments. This will allow us to grow cell bodies/proximal axons and distal axons in separate fluid environments, and quantitatively measure the extension of distal axons.

Our goals are to:

1. Establish an effective apoE purification system capable of producing at least 30 µg/ml of apoE.
2. Determine the concentration of compactin necessary to inhibit cholesterol synthesis in neurons by 50%.
3. Determine the effects of cholesterol synthesis inhibition on the extension of axons.
4. Determine the effects of apoE3 and apoE4 lipoprotein particles on axonal extension in the presence or absence of cholesterol synthesis inhibition.
5. Determine if cholesterol deficiency or possible apoE receptor-mediated signalling is the primary cause of apoE4 inhibitory effects.
6. Use a PCR-based protocol for genotyping LDL receptor mutant mice, which will allow us in the future studies to determine if the apoE isoform-specific effects are mediated via the LDL receptor.

CHAPTER II

Materials and Methods

2.1 MATERIALS

2.1.1 Cell culture materials

E. coli BL21 cells transformed with human apoE3 or apoE4 N-terminal (1-183) /pET plasmid were a generous gift from Dr. Robert O. Ryan (Children's Hospital Oakland Research Institute, Oakland, CA, USA) (Fisher et al. 1997).

Stable apoE3- and apoE4- transfected **neuroblastoma-2a (Neuro-2a)** cell lines were obtained from Dr. David E. Williams (State University of New York, Stony Brook, NY, USA) (DeMattos et al. 1998). For culturing neuro-2a cells we have used 75 cm² vented tissue culture flasks from BD Falcon, and 500 cm² vented triple layer tissue culture flasks from Nunclon. **Dulbecco's modified Eagle's medium (DMEM)** and **Ham's F-12 medium** nutrient mixtures, **fetal bovine serum (FBS)** and penicillin/streptomycin/amphotericin (10,000 units /ml penicillin G, 10,000 µg/ml streptomycin sulphate, 25µg/ml amphotericin) were purchased from Gibco-BRL. Medium was filter-sterilized using Millipore Stericup vacuum filtration devices.

Superior cervical ganglia were isolated from newborn Sprague-Dawley rats (Lab Animal Services, University of Alberta). Collagenase, trypsin, ascorbic acid (vitamin C), and cytosine arabinoside were purchased from Sigma. Teflon dividers were provided by Tyler Research Instruments, L-15 medium was purchased from Gibco-BRL, methyl cellulose from Sel-Win Chemicals LTD., **nerve growth factor (NGF)** from Cedarlane Laboratories Ltd., and rat serum was obtained from University of Alberta Lab Animal Services.

2.1.2 ApoE purification materials

Culture medium containing truncated N-terminal apoE was concentrated using (250 ml) stirred filtration devices with a 10 kDa **molecular weight cut off limit (MWCO)** from Filtron. Dialysis was performed using 12-14 kDa regenerated cellulose tubing from Fisher Scientific.

Heparin-Sepharose CL-6B resin was purchased from Amersham-Pharmacia Biotech. Gilson miniplus-2 peristaltic pump was used with a protein detector unit (single path monitor UV-1) from Amersham Pharmacia for circulation of medium and apoE detection.

For the monoclonal antibody column we have used cyanogen-bromide activated Sepharose 4B resin from Sigma. Monoclonal anti-human apoE antibody 1E was a generous gift from Dr. Linda Curtiss (The Scripps Research Institute, La Jolla, California, USA). For dialysis of eluate, we have used a 300 kDa MWCO Spectra/Por cellulose ester dialysis tubing from Fisher Scientific.

KBr density fractionation was done in polyallomer tubes from Seton Scientific. PD-10 (Sephadex G-25-M) size exclusion columns were from Amersham-Pharmacia Biotech.

Amicon Ultra Centrifugal Filter Device concentrators (YM-50 and 100 kDa MWCO with low-binding regenerated cellulose) were purchased from Millipore. All final products were filter-sterilized with sterile Millex-GV 0.22 µm low protein binding syringe mounted filters from Millipore.

2.1.3 Electrophoresis materials

Electrophoresis equipment was from Bio-Rad and most chemicals were from Gibco-BRL. Immobilon-P **polyvinylidene difluoride (PVDF)** membrane was purchased from Millipore. Polyclonal rabbit anti-rat apoE IgG (**J-115**) was produced in our laboratory by standard techniques, and the goat anti-rabbit IgG conjugated to **horseradish peroxidase (HRP)** was purchased from Pierce. **Enhanced Chemiluminescence (ECL)** detection reagents were purchased from Amersham-Pharmacia Biotech. For Western blot development we have used Kodak BioMax MR film and a Fuji RGII X-ray film developer.

2.1.4 Enzyme-linked immunosorbent assay (ELISA) materials

ELISA quantitation of apoE was performed in 96-well tissue culture plates from Fisher Scientific. Human recombinant apoE3 and apoE4 standards produced by baculovirus were purchased from Panvera. Detection reagent **3,3',5,5'-tetramethylbenzidine (TMB)** was purchased from Sigma.

2.1.5 Materials for experiments with sympathetic neurons

Mass cultures of sympathetic neurons from superior ganglia of one-day old rats were grown in 48-well tissue culture plates. Cholesterol and compactin (mevastatin) were purchased from Sigma. Compactin was dissolved in anhydrous ethanol to make a 10mM stock solution in methylcellulose-free L-15CO₂ medium. **Delipidated rat serum (dRS)** was obtained from University of Alberta Lab Animal Services. Protein assays were performed with **bicinchoninic acid (BCA)** assay kit from Pierce.

Silica Gel 60 **Thin Layer Chromatography (TLC)** plates were obtained from EM Science. Immunoblotting against rat tubulin was performed using mouse anti-rat α - and β -tubulin antibody mixture and goat anti-mouse HRP-conjugated antibody from Sigma.

2.1.6 Molecular biology materials

LDL receptor deficient mice were obtained from The Jackson Laboratories (mutant strain B6.129S7 – Ldlr^{tm1Her}) (Ishibashi et al. 1993), and maintained in a colony at the University of Alberta. For genotyping of the mice by **Polymerase Chain Reaction (PCR)**, we have used 3 different primers, as suggested by Jackson Laboratories:

IMR0014 (5'- AGG TGA GAT GAC AGG AGA TC - 3'),

IMR0046 (5'- ACC CCA AGA CGT GCT CCC AGG ATG A – 3'),

IMR0059 (5'- CGC AGT GCT CCT CAT CTG ACT TGT – 3').

The primers were synthesized at the University of Alberta. Proteinase K, PCR kit buffers, recombinant Taq polymerase and reagents were from Invitrogen.

2.1.7 Miscellaneous materials

All other standard laboratory materials were obtained from Fisher Scientific, Gibco-BRL, ICN Pharmaceuticals, Invitrogen, and Sigma. All solutions and buffers for laboratory use were made with de-ionized double distilled water (ddH₂O) from Milli-Q UF Plus water purification system (Millipore).

2.2 METHODS

2.2.1 Cell culture of *E. coli* cells producing N-terminal segment of apoE

Proteins with multiple domains that require extensive folding and post-translational modification are difficult to express in bacteria. Protein aggregation due to hydrophobic interactions has often been problematic. Since apoE contains amphipathic helices and is glycosylated, it was not until just recently that the full-length apoE was successfully expressed in *E. coli* cells. However due to the availability and effectiveness of expression of the N-terminal domain of apoE, previous experiments in our lab were conducted using a truncated form of apoE secreted by *E. coli* cells. This domain, when lipid-associated, is functional in binding to the receptor.

E. coli BL21 cells transformed with human apoE3 or apoE4 N-terminal (1-183) /pET plasmids were grown on LB plates in the presence of 50 ng/ml ampicillin. The culture method was described previously by Fisher et al. (Fisher et al. 1997). Briefly, a single colony of *E. coli* apoE3- and apoE4- expressing cells was used to inoculate 50 ml of 2x YT media, then grown overnight at 37°C to produce saturated cultures. These cells were transferred to 500ml of M9 minimal media at a 1:30 dilution and grown for 4 hours at 30°C to an optical density OD₆₀₀ of 0.600, at which time **isopropyl β-D-thiogalactopyranoside (IPTG)** was added to a final concentration of 1 mM in order to induce apoE production. The culture was then incubated for another 4 hours at 30°C to allow for apoE synthesis.

The medium was collected and centrifuged at 4°C in a JA-14 rotor at 10,000 x g for 10 minutes in order to pellet the cells. The supernatant, which contains the apoE product, was then concentrated by centrifugation in

Filtron 10 kDa MWCO stirred cell to a volume of 30 ml. This product was then dialyzed against 20 mM Na₂HPO₄, 150 mM NaCl, pH 7.0 in 12-14 kDa MWCO dialysis tubing at 4°C overnight.

2.2.2 Cell culture of Neuro-2a cells secreting full-length human apoE

Neuro-2a cell lines stably expressing human apoE3 and apoE4 (DeMattos et al. 1998) were maintained in a 37°C, 95% air / 5% CO₂ humidified incubator, in a 1:1 mixture of DMEM and F-12 media supplemented with 10% heat-inactivated FBS (10% FBS, DMEM-F12), as suggested by DeMattos et al. (DeMattos et al. 2001). Cells were grown in 75 cm² vented Falcon tissue culture flasks and split 1:10 every 3 days.

In order to harvest secreted full-length apoE3 and apoE4, 90% confluent cells were split 1:20 into 100 ml of (10% FBS, DMEM-F12) medium and plated in 500 cm² vented Nunc triple layer flasks. Each flask of cells was grown for 3 days, washed, and incubated for 15 minutes with 50ml of DMEM-F12 (1:1) medium lacking FBS to remove exogenous apoE, and then incubated for 48 hours with 100 ml of DMEM-F12 medium lacking FBS to allow for apoE secretion. The harvested apoE conditioned medium was then centrifuged at 2000 x g in a tabletop centrifuge at 4°C for 15 minutes to pellet the cells, and supernatant containing apoE was collected. The parental untransfected Neuro-2a cell line, which does not secrete apoE particles, was treated in parallel with transfected cells and its medium collected for control purposes.

These minimally lipidated full-length apoE3 and apoE4 particles have similar phospholipid and cholesterol composition, and have previously been shown to exhibit isoform-specific effects on neurite outgrowth of

Neuro-2a cells (DeMattos et al. 2001). Aside from phospholipids, these particles also contain one molecule of cholesterol per molecule of apoE, however, unlike LDL and HDL, these particles contain no triacylglycerols and cholesteryl esters.

2.2.3 Isolation of rat sympathetic ganglion neurons

Superior cervical ganglia neurons were dissected from one-day-old Sprague-Dawley rats and cultured using an established and previously reported method (Campenot 1992). Briefly, the rats were decapitated, ganglia were removed by dissection and enzymatically dissociated first by a 25 minute treatment with collagenase at 37°C, and then by a 5 minute trypsin treatment, after which, the neurons were released with the help of gentle trituration through a glass Pasteur pipette.

2.2.4 Culture method for sympathetic neurons

The rat sympathetic ganglion neurons were grown in compartmented culture dishes which contain teflon dividers. These three-compartment dividers permit culture of cell bodies and axons in separate fluid environments, and allow us to conduct axon elongation experiments (Campenot 1982). **Figure 2.1** shows a typical three-compartment dish used to culture rat sympathetic neurons.

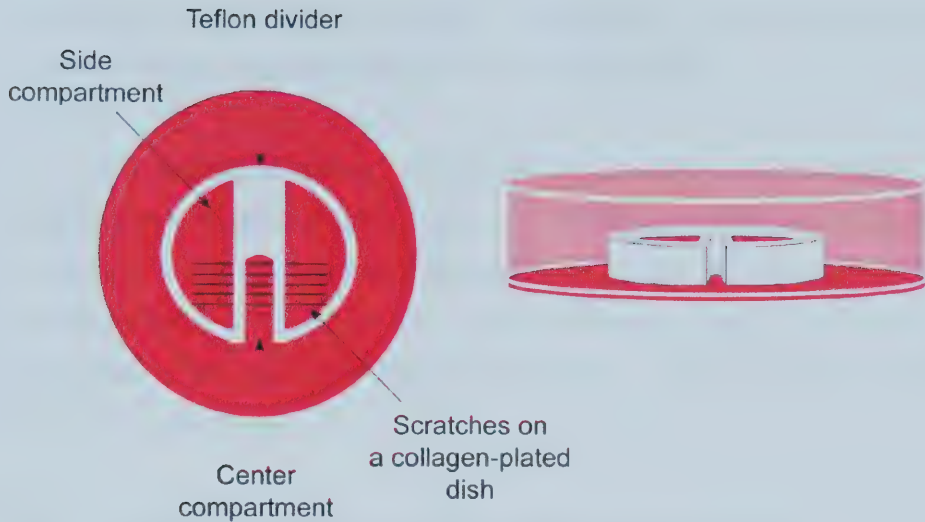


Figure 2.1 : Compartmented culture dish. A Teflon divider allows us to plate cell bodies/proximal axons of neurons in the center compartment, and measure distal axon extension in the side compartments. The scratches create 21 channels which force axons to extend in a straight line. Silicone grease covers the bottom of the divider and seals it to the dish, thus preventing medium leakage among the compartments. The axons cross from the center into the side compartments, where there is a greater NGF concentration.

Briefly, a series of 35 mm culture dishes were coated with collagen to provide a surface for neuron attachment. Twenty-one parallel scratches were made on the surface of each dish in order to provide direction for axonal elongation. Neurons were plated at a density of 0.4 ganglia/dish. In the center compartment, where the cell bodies/proximal axons were located, the L-15CO₂ methylcellulose base medium was supplemented with 2.5% rat serum and 1 mg/ml vitamin C. During the first week of culture, 10 ng/ml of NGF was added to the cell body compartments to stimulate neuron growth and survival. Furthermore, 10 mM cytosine arabinoside was added

to prevent non-neuronal cell growth. Medium in the axon-containing compartments was supplemented with 50 ng/ml of NGF.

For some experiments, neurons were grown in collagen-treated 48-well plates at a density of 0.5 ganglia/well. These mass cultures of neurons were grown in L-15CO₂ methylcellulose base medium supplemented with 50 ng/ml of NGF, 2.5% rat serum, 1 mg/ml vitamin C, and 10 mM cytosine arabinoside. The cells were cultured for 7 days, at which time they were used in the experiments.

2.2.5 ApoE purification by heparin-Sepharose affinity chromatography

The conditioned medium from apoE-transfected neuro-2a cells was passed through a 10 ml heparin-Sepharose CL-6B column, washed with PBS (2.7 mM KCl, 1.5 mM KH₂PO₄, 137 mM NaCl, 8 mM Na₂HPO₄-2H₂O, pH 7.4) and eluted with 1 M NaCl PBS. The eluate was then dialyzed against PBS in 12-14 kDa MWCO dialysis tubing to remove salts. In the case of the N-terminal truncated apoE, the product was flash frozen at -80°C, lyophilized and stored at -20°C until further use, while the full-length apoE product was further subjected to antibody column purification.

2.2.6 ApoE purification by immunoaffinity chromatography with monoclonal apoE antibody

To prepare the column, we dissolved 1 g of cyanogen-bromide activated Sepharose 4B in 1 mM HCl and washed the resin with 400 ml of 1 mM HCl over a Büchner funnel. After rinsing the gel with 500 ml of distilled water, we dissolved it in the **coupling buffer** (0.1 M NaHCO₃, 0.5 M NaCl, pH 8.3 - 8.5) and transferred it to a solution of PBS containing 10 mg of

monoclonal anti-human apoE antibody 1E (DeMattos et al. 1998). The antibody was allowed to bind the resin overnight at 4°C on a rotary shaker. The resin was then washed with a coupling buffer, and incubated with a blocking solution (1 M ethanolamine, pH 8.0) on a rotary shaker overnight at 4°C to block un-reacted groups. The resin was then washed over a funnel with a basic coupling buffer and then with an acidic buffer (0.1 M acetic acid, 0.5 M NaCl, pH 4), in a consecutive five-time cycle to remove the blocking solution. The resin was packed into a column, washed with 1M NaCl and kept at 4°C in PBS buffer containing bacteriostat (0.02% sodium azide).

To purify apoE lipid-associated particles, 100 ml of apoE conditioned medium from the transfected Neuro-2a cells was circulated at 4°C overnight through a monoclonal antibody column at a rate of 1 ml/min in order to allow for binding of apoE to the antibody. The column was then washed with 100 ml of PBS to remove non-binding medium contents. ApoE particles were eluted with 20 ml of 3 M sodium thiocyanate, and dialysed against 4 cycles of 4 L sterile PBS at 4°C, using a 300 kDa MWCO cellulose ester dialysis tubing (Spectra/Por).

2.2.7 ApoE purification by KBr density gradient ultra-centrifugation of apoE lipid particles

To purify apoE lipid-associated particles using a method of KBr density gradient ultracentrifugation, as previously described by DeMattos et al., we used 4 ml of apoE3 conditioned medium from neuro-2a cells, adjusted its density with KBr to 1.20 g/ml, underlaid it with 4 ml of medium with density of 1.34 g/ml and overlaid it with 4 ml of medium with density of 1.10 g/ml in six 12 ml polyallomer tubes (DeMattos et al. 1998). We used a SW40 rotor at 37,600 rpm for 48 hours at 4°C to centrifuge the gradients,

and twelve 1 ml fractions were collected from top to bottom. The density of each fraction was measured by weighing 1 ml of each sample, and the apoE content of each fraction was determined by ELISA. The product was dialyzed and purity assessed by Western blot and silver stained gels.

2.2.8 ApoE purification by size exclusion chromatography

In some experiments, in order to transfer apoE particles from the apoE conditioned DMEM/F12 medium into L-15 base medium which is used for culturing neurons, we used size exclusion chromatography. Size exclusion columns (Sephadex G-25) were equilibrated with 25 ml of L-15 methylcellulose-free base medium, apoE conditioned medium was filtered through the column, and the final product in L-15 medium was collected at the bottom of the column.

2.2.9 ApoE purification by ultra-filtration centrifugation

In some experiments we utilized Amicon 15 ml concentrators with a 50 or 100 kDa MWCO for simultaneous apoE concentration, purification and medium exchange. To purify apoE we centrifuged 100 ml of apoE containing DMEM-F12 conditioned medium at 2000 x g in a 4°C refrigerated tabletop centrifuge. Since the concentrators used held only 15 ml volume, several consecutive centrifugation steps were repeated to reduce the volume from 100 ml to 0.5 ml, after which the medium was washed with 30 ml of PBS, and ultimately with 15 ml of L-15 methylcellulose-free base medium. The final concentrated product of 1 ml was collected and filter-sterilized with sterile Millex 0.22 µm low protein binding syringe mounted filter. ELISA was used as a final step to determine the apoE concentration.

2.2.10 Immunoblotting of apoE

The purity of apoE was analyzed using SDS PAGE. Samples were boiled for 10 minutes in **sample buffer** (12 mM Tris, 5% glycerol, 4% SDS, 0.02% bromophenol blue, pH 6.8) containing 5% β -mercaptoethanol, then run on a 12.5% SDS-polyacrylamide gel for 1 hour at 60 mA, and transferred onto an Immobilon-P polyvinylidene difluoride (PVDF) membrane (Millipore) for 1.5 hours at 60 V or at 4°C overnight at 15 V.

Immunoblotting was performed first by blocking the membrane with 7% milk in PBS-T (0.05% Tween-20 in PBS), then probing with a primary rabbit anti-rat apoE antibody (J-115) diluted at 1: 2,500 in 7% milk PBS-T. Next, secondary goat anti-rabbit IgG conjugated to horseradish peroxidase was applied at 1:10,000 dilution in PBS-T. The membrane was washed in PBS-T for 30 minutes minimum between incubations. The detection was performed with enhanced chemiluminescence (ECL) reagents.

2.2.11 Silver and Coomassie Blue staining of gels

Silver staining of gels was performed by first fixing the gel in **destaining solution** containing water : methanol : acetic acid (50:40:10) overnight. The gel was rinsed with water twice for 15 minutes, and sensitized with 0.02% $\text{Na}_2\text{S}_2\text{O}_3$ solution for 2 minutes. After rinsing for 1 minute with water, the gel was stained with **silver solution** (0.2% AgNO_3 , 1 mM formaldehyde) for 25 minutes and rinsed with water for 1 minute. **Developing solution** (4% Na_2CO_3 , 6 mM formaldehyde, 20 μM $\text{Na}_2\text{S}_2\text{O}_3$) was used to develop the gel. The gel was then incubated in destaining solution for 5 minutes, rinsed with water and stored.

Coomassie Blue staining was performed first by staining the gel overnight in Coomassie Brilliant Blue G-250 (0.6% in destaining solution), rinsing with water and washing in destaining solution until developed.

2.2.12 Enzyme-linked immunosorbent assay (ELISA)

In order to quantitatively analyze apoE concentration, we established an ELISA protocol using human recombinant apoE standards. For a standard curve we used a stock solution of 10 µg/ml of human apoE diluted in the range of 0, 6.25, 12.5, 25, and 50 ng per well. Into 96-well tissue culture treated plates we loaded 100 µl of sample per well, diluted 1:100 or 1:1,000 in PBS-T, depending on whether the sample was from the cell medium or from the purified concentrated product. The samples were incubated for 1-2 hours at room temperature, washed gently three times with PBS-T, and blocked for 1 hour with 200 µl of 7% milk PBS-T solution at room temperature. The wells were washed three times with PBS-T, and 100 µl of J-115 primary polyclonal rabbit anti-rat apoE antibody (1: 2,500) in PBS-T was applied to each well and incubated overnight at 4°C. After washing the wells three times with PBS-T, 100 µl of secondary goat anti-rabbit HRP conjugated antibody (1:10,000) in PBS-T was applied and incubated for 1 hour at room temperature. The wells were then washed five times with PBS-T. To develop the plate, 150 µl of 3,3',5,5' – tetramethylbenzidine (TMB) was added per well, and the samples were incubated for 30 minutes at room temperature. Color development was scanned at a wavelength of 650 nm.

2.2.13 Compactin inhibition of cholesterol synthesis in neurons

To determine a dose of compactin necessary to inhibit cholesterol synthesis in neurons by 50% we incubated mass cultures of neurons with

[¹⁴C]acetate in the presence of different compactin (mevastatin) concentrations and measured the incorporation of [¹⁴C]acetate into cholesterol (Posse de Chaves et al. 1997).

Mass cultures of sympathetic neurons grown in a 48-well plate for 6 days were washed and incubated overnight with L-15CO₂ base medium containing 2.5% delipidated rat serum, 1mg/ml vitamin C, 50 ng/ml and different concentrations of 0, 10, 50, 100, 500, 1000 nM of compactin. The condition with 0 nM compactin was used as a control, and since compactin was dissolved in a dilute solution of ethanol, we supplemented the control medium with the equivalent amount of ethanol to match the other conditions (0.01% v/v). This 24 hour pre-incubation with compactin in a lipid-free medium allowed us to establish a range of inhibition of cholesterol synthesis prior to applying [¹⁴C]acetate. The medium was then replaced with fresh medium containing compactin supplemented with [¹⁴C]acetate (5 µCi/ml). After a 24 hour pulse incubation, each well was washed and incubated twice with 1 ml PBS containing methylcellulose, for 10 minutes at 37°C. A final 5 minute wash with PBS at room temperature ensured the entire unincorporated label and medium were removed. The cells were first harvested with 100 µl PBS containing 0.1% deoxycholate and EDTA, and the wells were washed again with 150 µl PBS to a total combined volume of 250µl. Protein concentration was determined for each sample using the bicinchoninic acid (BCA, Pierce) assay kit at 60°C.

Lipids were extracted from the cells by placing the sample into a glass screw-cap tube, adding hexane/iso-propanol (3:2), vortexing the mixture for 2 minutes, and centrifuging for 5 minutes at 2000 rpm in a tabletop centrifuge to separate the phases. The upper organic phase was dried under nitrogen gas. To each sample we added 10µl of 10mg/ml unlabelled unesterified cholesterol as a carrier and 80 µl of chloroform. The

extract was then applied to a TLC plate (silica gel G60), which was placed in a glass tank containing heptane : isopropyl ether : acetic acid (60:40:4). The bands corresponding to separated lipids were visualized with iodine and the band corresponding to authentic cholesterol standard was scraped. The samples were put into scintillation vials with 5 ml of scintillation fluid and radioactivity was counted.

2.2.14 Axotomy and standard experimental setup for measuring axonal extension in compartmented dishes

After culturing sympathetic neurons for 7-10 days, the distal axons were removed by axotomy. This procedure involves the amputation of distal axons in the side compartments, which is achieved by three consecutive washes using sterile distilled water and a 22-gauge syringe. The osmotic pressure of distilled water and the mechanical force of the stream are sufficient to wash away the axons without damaging the cell bodies in the center compartment. This is marked as day zero of the experiment and at this time fresh medium is supplied to the neurons. For measuring axonal extension we used a Nikon microscope whose stage was connected to a MD2 microscope digitizer (Minnesota Datametrics). At 24 hour intervals, the axonal extension was measured, starting from the divider barrier that separates the center compartment and the side compartment, to the tip of the farthest extending distal axon, adjusting for the irregularities in the grease, as demonstrated by Campenot (Campenot 1992). Axonal extension was measured in triplicate dishes for each experimental condition, with thirty-two tracks per dish (n=96), and data were expressed as means and standard errors of the mean.

2.2.15 Inhibition of axonal extension by compactin

We used compactin to determine how the inhibition of cholesterol synthesis affects extension of axons in compartmented cultures of rat sympathetic neurons. After axotomy, the center compartments were supplemented with fresh L-15CO₂ methylcellulose medium containing delipidated rat serum and vitamin C, and the axonal compartment was supplemented with 50 ng/ml NGF. Concentrations in the range of 0 – 1,000 nM compactin were added to center compartments. As a control we used dishes cultured under normal conditions with rat serum and vitamin C in the cell bodies/proximal axons compartment and 50 ng/ml NGF in the distal axons compartment. The cultures were grown for 4-5 days and axonal extension was measured every 24 hours.

2.2.16 Effects of apoE on axonal extension

ApoE lipid-containing particles secreted by Neuro-2a cells were concentrated, purified and applied to compartmented cultures of neurons in the presence of delipidated rat serum. Prior to incubation, the apoE concentration was determined by ELISA and adjusted to 30 µg/ml. The medium in the center compartment was supplemented with 50 nM compactin to inhibit cholesterol synthesis. After axotomy, axonal elongation was measured over a period of 4-5 days. All conditions were applied to triplicate dishes. **Figure 2.2** illustrates the steps of this procedure.

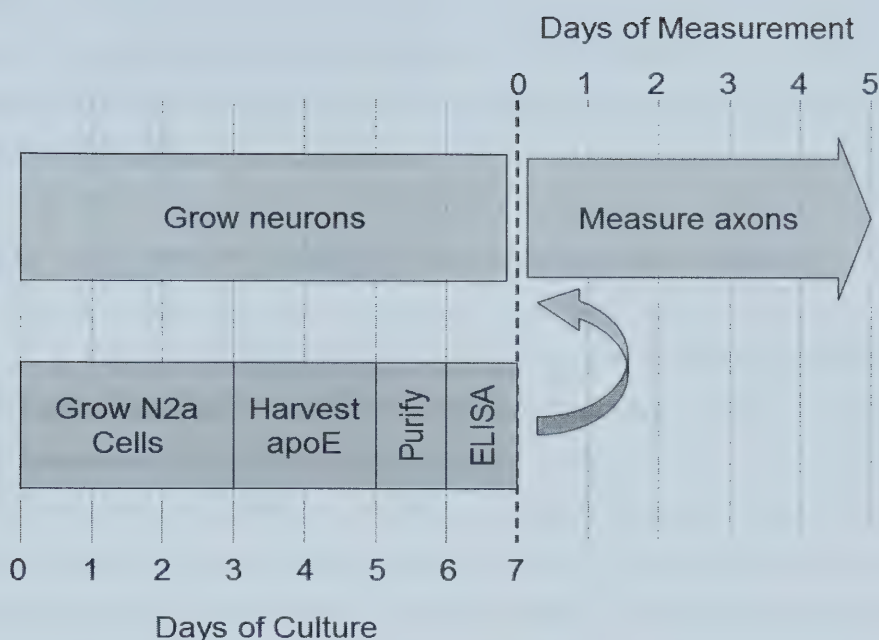


Figure 2.2 : Experimental setup for measuring the effects of apoE on axonal extension. Neurons were grown in compartmented dishes for seven days, during which time apoE was isolated and purified from Neuro-2a cells as indicated in section 2.2.2. Dashed line indicates axotomy and the start of the experiment.

2.2.17 Immunoblotting against axonal α - and β -tubulin

Cellular material in axonal side compartments was harvested in triplicate dishes with 2x sample buffer (24 mM Tris, 10% glycerol, 8% SDS, 0.04% bromophenol blue, pH 6.8); samples were boiled in the presence of β -mercaptoethanol, and proteins were run on a 10% SDS-PAGE gel. The proteins were transferred onto a PVDF membrane, and the membrane was blocked with 5% milk in PBS-T, probed first with mouse anti-rat tubulin antibody (1:1,000 each α - and β - tubulin) and then with goat anti-mouse IgG antibody conjugated to HRP (1:10,000). The membrane was washed with

PBST and exposed to ECL reagent for film development. The exposed film results were analysed and compared by densitometry (measuring pixel density per area of film) using BioRad Molecular Imager FX scanner and Quantity One quantitation software.

2.2.18 PCR-based system for genotyping LDLR ^{-/-} mice

A PCR-based method for genotyping LDLR ^{-/-} mice has previously been established and shown to amplify a 400 bp wild-type product and a 200 bp mutant product (Gaw et al. 1995). In our study, a similar method was implemented using a modified protocol obtained from Jackson Laboratories. The two protocols utilize different PCR cycling conditions and different primers for amplifying the mutant allele. While the former protocol by Gaw et al. amplified a 200 bp mutant product, the protocol used in this study amplified an 800 bp mutant product. Since mice were obtained from Jackson Laboratories, we have opted to use their suggested genotyping protocol.

Murine genomic DNA was isolated by digesting a 2 mm fragment of the mouse tail with fungal **proteinase K**, a non specific serine protease. Tails were incubated overnight at 56°C, with 500 µl of 0.856 µg/µl of proteinase K in buffer (20 mM Tris-HCl, 50 mM EDTA, 100 mM NaCl, 0.5% SDS, pH 7.5). Once the tails were digested, three organic extractions were performed. First we added 500 µl of phenol, mixed the contents by inversion, centrifuged it at 13,000 rpm for 1 minute, and transferred the upper aqueous phase to a new tube. To this aqueous phase we added 500 µl of phenol/ chloroform (1:1), mixed it by inversion, centrifuged it at 13,000 rpm for 1 minute and collected the upper aqueous phase. After transferring the aqueous phase to a fresh tube, 500 µl of chloroform was added, the contents were mixed by inversion, the tube was centrifuged at 13,000 for 1

minute, and the upper aqueous phase was transferred to a new tube. To this aqueous phase we added 300 µl of isopropanol. The tube was mixed and centrifuged for 15 minutes at 4°C to pellet the DNA. The pellet was washed with 500 µl of ice-cold 70% ethanol, and centrifuged for 15 min at 4°C. Ethanol was removed, the pellet was briefly dried and 100 µl of sterile TE buffer (10 mM Tris, 1mM EDTA, pH 8.0) was added. The tubes were incubated overnight at 37°C to resuspend the DNA.

For genotyping wild-type and LDLR ^{-/-} mutant mice using PCR, we used three primers as suggested by Jackson Laboratories, namely IMR0014 (5'- AGG TGA GAT GAC AGG AGA TC - 3'), IMR0046 (5'- ACC CCA AGA CGT GCT CCC AGG ATG A – 3'), and IMR0059 (5'- CGC AGT GCT CCT CAT CTG ACT TGT – 3'). Two PCR reactions were set up for each DNA sample, one for detecting a 400 bp wild-type product (using primers IMR0059 and IMR0046) and the other for detecting an 800 bp mutant product (using primers IMR0014 and IMR0046). The primers were dissolved in **TE buffer** (10 mM Tris, 0.1mM EDTA, pH 8.0), and their concentration determined with a spectrophotometer using a wavelength of 260 nm.

In a 50 µl reaction, we added 1x buffer, 0.4 mM dNTP mix, 2 mM MgCl₂, 0.1 µM of each primer, 5 µl of DNA, 2.5 units of Taq polymerase. PCR cycle conditions were as shown in **Table 2.1**.

STEP	TEMP	TIME	note
1	94°C	3 min	
2	94°C	35 sec	
3	64°C	45 sec	- 0.5°C per cycle
4	72°C	1 min	Go to step 2, 12 times
5	94°C	35 sec	
6	58°C	30 sec	
7	72°C	1 min	Go to step 5, 25 times
8	72°C	2 min	
9	4°C		

Table 2.1 : PCR cycling conditions. A modified version of the protocol suggested by Jackson Laboratories, in which elongation time was increased in order to improve the yield of the mutant product.

2.2.19 Agarose gel electrophoresis of PCR products

To each PCR product tube, 9 µl of 6x **loading buffer** (0.25% bromophenol blue, 0.25% xylene cyanol, 50% glycerol) was added, and samples were run on a 1.5% agarose gel alongside a 100 bp ladder marker. The wild-type product was expected to have a single 400 bp band, the LDLR -/- mutant homozygote was expected to have a single 800bp band, and the heterozygote was expected to have both bands present. The DNA bands were visualized using ethidium bromide and ultraviolet light.

CHAPTER III

Results

3.1 **BACKGROUND**

In order to apply apoE3 and apoE4 to neurons and measure the effects on axonal extension, apoE had to be isolated, purified and concentrated to 30 µg/ml. To achieve this we used two apoE models. The first model involved the use of an N-terminal domain (residues 1-183) of human apoE, expressed in *E. coli* cells. This domain of apoE contains a receptor binding site and is capable of forming functional discoidal complexes with lipids such as dimyristoylphosphatidylcholine (Fisher et al. 1997). The advantage of this model is the ability to efficiently express the recombinant protein in large amounts.

The second model involved the purification of the full-length human apoE secreted by transfected Neuro-2a cells. The advantage of this model is that it more closely resembles physiological features of the lipoprotein particles that would interact with neurons in vivo, not only with regards to the complete structure of the apoE but also the lipid composition of the particles. Cholesterol and phospholipids are present on the apoE particles (DeMattos et al. 2001). However, compared to plasma HDL or LDL particles, the apoE particles are relatively lipid poor.

We explored the use of several purification methods, such as heparin-Sepharose column chromatography, a monoclonal anti-apoE antibody affinity column, size exclusion chromatography, ultra-centrifugation density fractionation, and ultra-filtration centrifugation.

3.2 PURIFICATION OF APOLIPOPROTEIN E

3.2.1 Purification of the N-terminal apoE fragment secreted from *E. coli* cells

E. coli cells transfected with a truncated N-terminal form of apoE3 or apoE4 were cultured to secrete apoE into the medium. Upon isolation and purification by a heparin-Sepharose column, the purity was assessed by immunoblotting. The truncated N-terminal of apoE has a predicted 21 kDa molecular mass (Fisher et al. 1997). However, on SDS-PAGE the molecular mass of the protein was in the range of 28 kDa. **Figure 3.1** shows a typical Western blot example of this purification process. For comparison, samples are shown from the medium (cell supernatant) prior to purification and from the purified product for each apoE3 and apoE4.



Figure 3.1 : Western blot of a truncated N-terminal apoE fragment secreted by transformed *E. coli* cells. The apoE was purified using heparin-Sepharose chromatography, and run on a 12.5 % SDS-PAGE gel. Equal volumes of samples were loaded in each lane. “*Medium*” indicates the supernatant directly obtained from the culture medium, and “*Eluate*” is the product obtained after purification. Arrow indicates the position of apoE.

In order to examine the purity of apoE products, we stained an identical gel with silver stain. Silver stain is a non-specific and yet sensitive protein detection system able to detect the overall protein content. **Figure 3.2** shows that the heparin-Sepharose purification system was not able to specifically isolate apoE, as several protein bands are visible, either due to *E. coli* impurities, or apoE aggregation. The pronounced 28 kDa apoE3 band detected by the Western blot was modestly detected by the silver stain; however the apoE4 band, which was not as concentrated, appeared barely visible in the silver stain, indicating that apoE4 expression was slightly lower than that of apoE3, and that at this concentration the apoE content was below the detection limit of the stain.

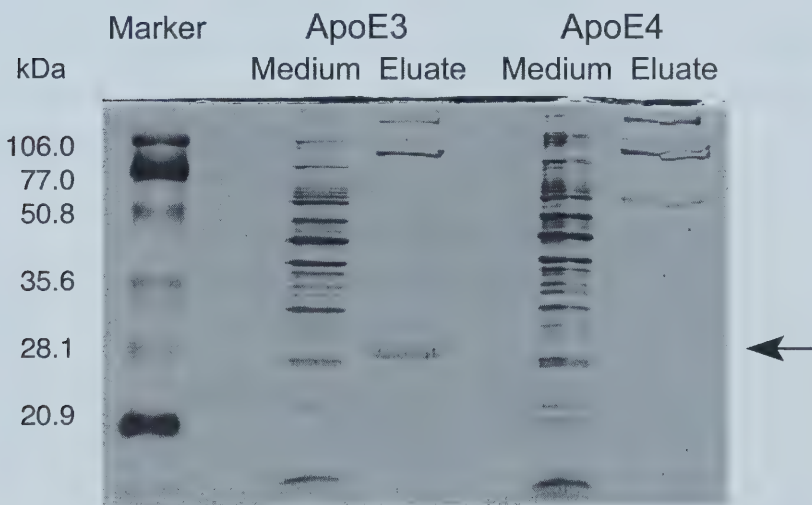


Figure 3.2 : Silver stain of a truncated N-terminal apoE fragment secreted by transformed *E. coli* cells. This gel was run in parallel with the one in figure 3.1 and shows the overall protein content of the unpurified cell culture supernatant ("Medium") and of the purified and concentrated product ("Eluate"). Arrow indicates the position of apoE.

3.2.2 Purification of the full-length human apoE3 and apoE4 secreted by Neuro-2a cells

Since the purity and concentration of the truncated apoE did not meet our expectations, we decided to switch to a more physiological model of apoE. Full-length human apoE secreted in a lipidated form by Neuro-2a cells was isolated using several techniques.

Heparin-Sepharose column purification involved running the apoE-containing medium overnight through the pump and into a protein detector device connected to a printer. **Figure 3.3** shows the real-time readout of the protein content flowing through the detector. The medium was allowed to drain through the column, and the non-binding proteins and medium were washed away, by washing the column with several column volumes of PBS. After the washing stage, a 3 M sodium thiocyanate solution was introduced into the column to elute apoE. This high-salt elution step was then followed by dialysis in PBS with 12-14 kDa MWCO tubing.

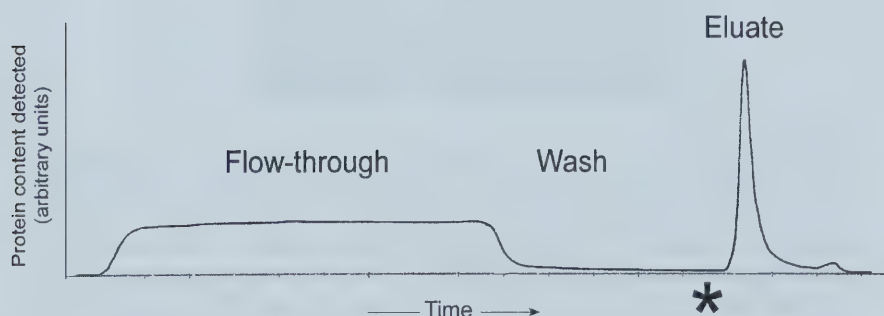


Figure 3.3 : Real-time diagram of an apoE elution from a heparin-Sepharose column. The “*Flow-through*” contains DMEM-F12 medium used to culture Neuro-2a cells, non-binding protein content and small traces of non-bound apoE. Washing with PBS purified apoE and 3 M sodium thiocyanate (*) introduction released apoE from the column (“*Eluate*”). The height of the peaks indicates a relative protein content flowing through the detector at a given time.

Heparin-Sepharose column purification allowed us to concentrate apoE. However, silver stain of the gel indicated that impurities were still present. This was in part due to an ability of heparin-Sepharose to bind, aside from apoE, a multitude of other proteins. **Figure 3.4** shows an example of heparin-Sepharose purification of apoE lipid particles.

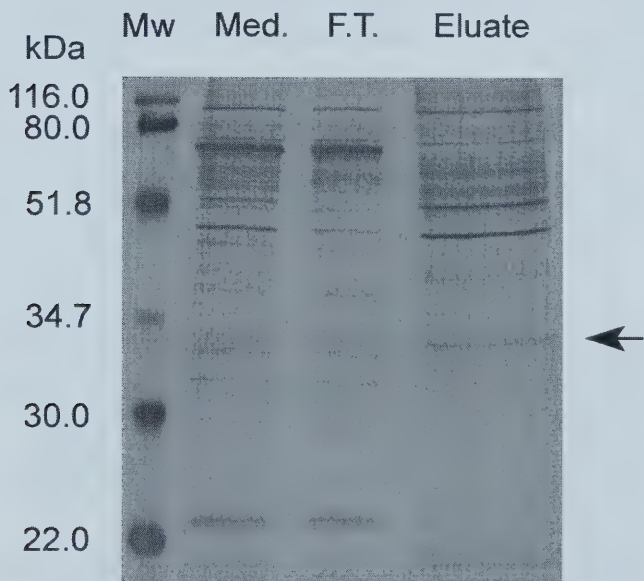


Figure 3.4 : Heparin-Sepharose purification of apoE3 particles. Full-length apoE lipid-associated particles were purified on a column then run on a 12.5 % SDS PAGE gel. Equal sample volumes were loaded in each lane. “Medium” is directly obtained from the culture medium, “F.T.” (flow-through) indicates non-binding proteins and “Eluate” is the product obtained after high-salt elution. Arrow indicates the position of apoE.

We have also tried to perform a serial elution with increasing salt concentrations. However, instead of separating apoE from other proteins, this procedure only diluted the apoE content.

3.2.3 Monoclonal antibody column purification of apoE

Due to our inability to sufficiently purify and concentrate apoE using heparin-Sepharose, we attempted to purify apoE using a monoclonal anti-apoE antibody column. Medium collected from apoE3-secreting Neuro-2a cells was re-circulated through the column overnight to allow apoE particles to bind to the antibody column. The column was washed with PBS, and the apoE particles were eluted with 20 ml of 3M sodium thiocyanate. **Figure 3.5** shows a Western blot of the purified product.

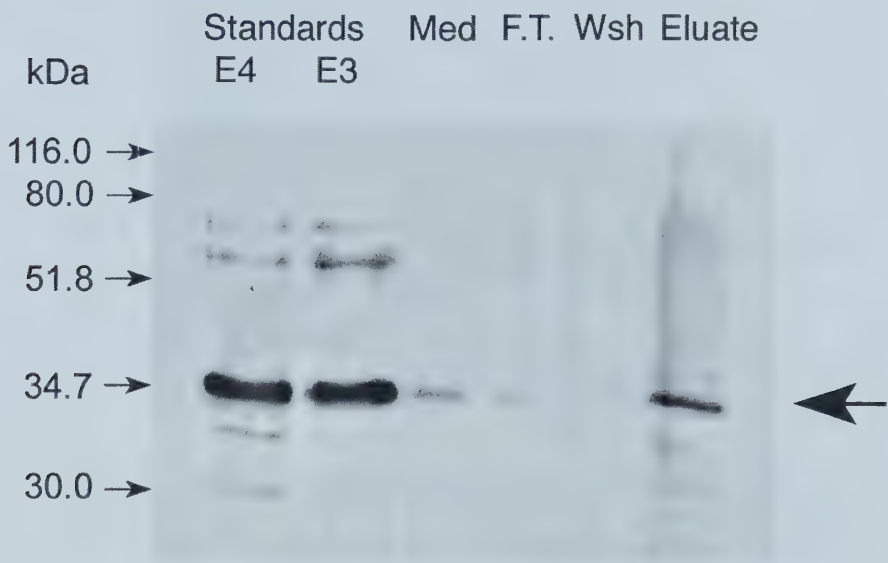


Figure 3.5 : Western blot of apoE purified with an antibody column. The apoE-containing medium (“Med”) was run through the column and the non-binding flow-through (“F.T.”) material was washed (“Wsh”) with PBS. ApoE was then eluted as a purified product. Equal volume of each sample was run on a 12.5% SDS-PAGE gel. Stock solutions of apoE3 and apoE4 standards used for ELISA were also run in comparison. Arrow indicates the position of apoE.

In order to further assess the purity of the eluted apoE, we stained a similar gel with Coomassie blue, as indicated in **Figure 3.6**. Although the antibody column generated a product of higher purity than the heparin-Sepharose column, the small volume of the antibody column did not allow for large quantities of apoE to be purified.

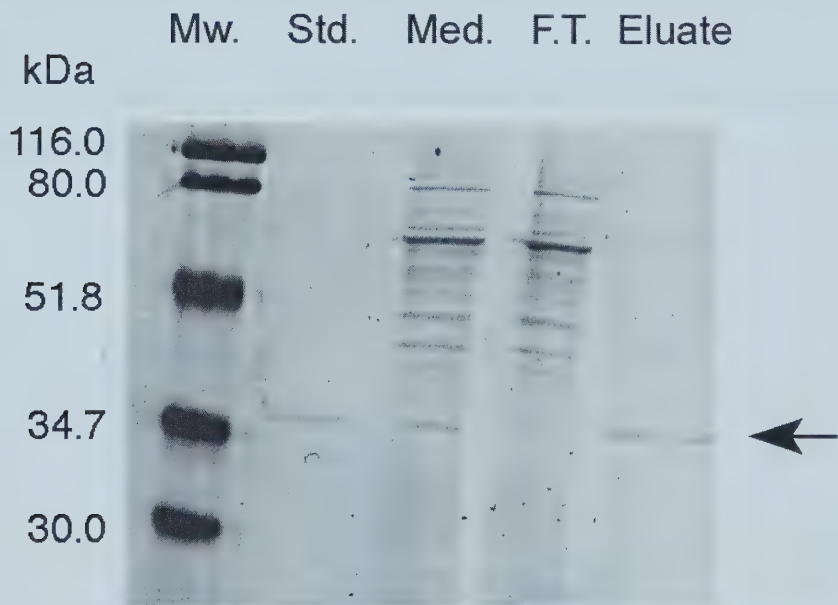


Figure 3.6 : Coomassie blue stain of apoE purified with an antibody column. This 12.5% SDS PAGE shows different stages of purification of apoE3 using a monoclonal anti-apoE antibody column. “Mw” - molecular weight standards, “Std”- apoE standard, “Med”- unpurified conditioned medium, “F.T.”- non-binding medium contents, “Eluate”- purified apoE product. Arrow indicates the position of apoE.

3.2.4 ApoE yield decreases during each purification step

During the purification of apoE, every step lowered the total amount of apoE protein recovered, and more complicated procedures involving extensive transfer, filtration and dialysis of the medium proved to be inefficient. **Figure 3.7** shows that if 100 ml of apoE-containing medium was circulated overnight, the antibody column was able to bind about 75% of total apoE found in the medium (600 μ g of total 800 μ g was bound to the column and eluted). Once that eluate was further concentrated by ultra-filtration and dialyzed to remove salts, the total amount of apoE recovered was significantly reduced (about 20% yield).

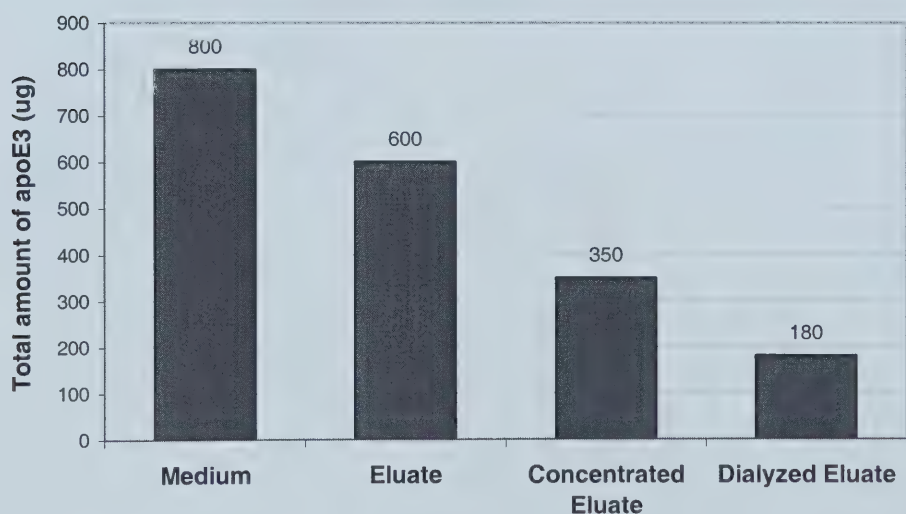


Figure 3.7 : Progressive loss of apoE3 during the purification process. The medium from Neuro-2a cells was purified with the antibody column. The eluate was then further concentrated with Millipore YM-50 concentrators and dialyzed with the 300 kDa MWCO dialysis tubing. Yield is expressed as the total amount of apoE recovered during each step of the purification of 100 ml of medium.

3.2.5 Purification of apoE by KBr density ultracentrifugation

Since apoE is associated with a lipid particle, we decided to utilize this property to our advantage by using its density for purification purposes. Ultracentrifugation in a density gradient allowed us to isolate the lipid particles from the other non-lipid bound proteins. However this purification method required extensive dialysis to remove the KBr and make apoE particles suitable for experiments with the neurons. **Figure 3.8** shows the apoE lipid particle distribution on a density gradient. Most of the particles were found in the density range of 1.19 to 1.27 g/ml, which is in accord with previously published data (DeMattos et al. 1998).

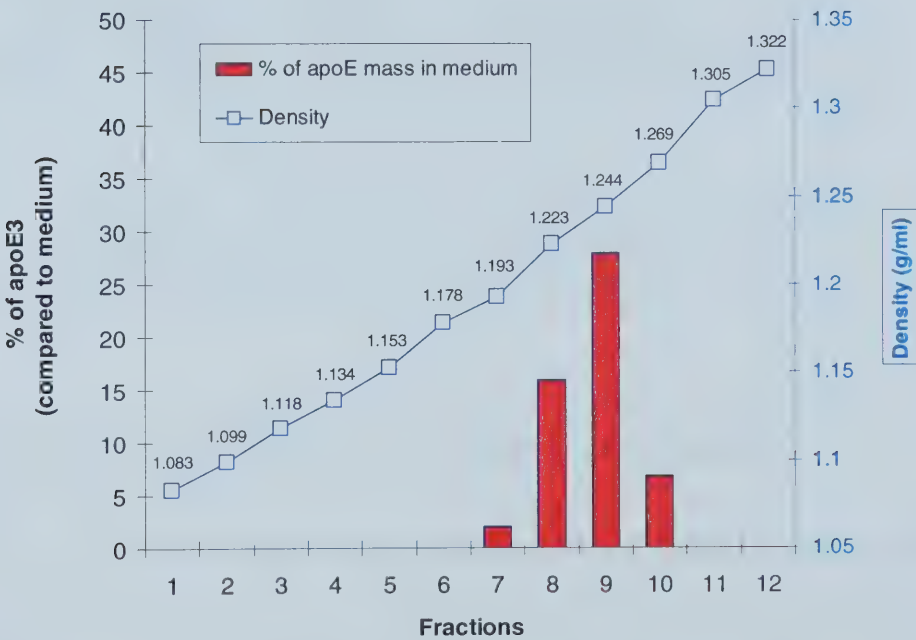


Figure 3.8 : Purification of apoE by KBr density gradient ultracentrifugation. ApoE containing medium was centrifuged and separated into 12 fractions on a KBr density gradient. The samples were analyzed by ELISA to determine the apoE content of each fraction. Data are displayed as percentage of total apoE compared to the conditioned medium. Fraction 1 indicates top of the tube, while fraction 12 indicates the bottom.

To confirm the presence of apoE in each fraction obtained by centrifugation, and to assess the purity of the product, we have analyzed the samples by SDS-PAGE. Immunoblotting confirmed the results obtained by ELISA, as most of the apoE was found in fractions 7 through 10, as illustrated in **Figure 3.9**.

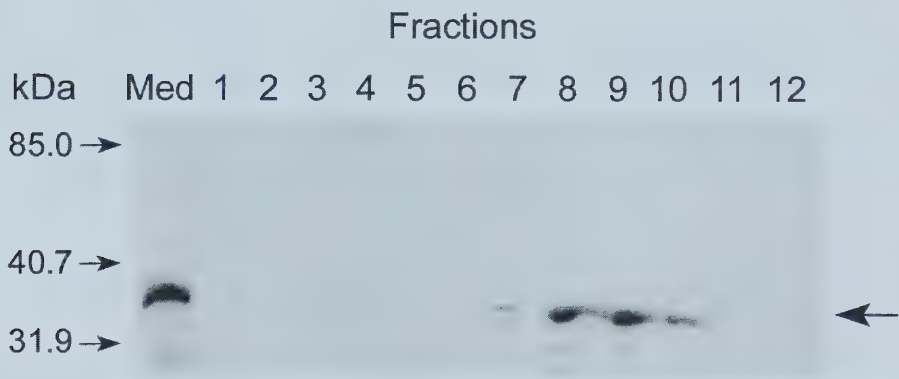


Figure 3.9 : Western Blot analysis of apoE3 density fractionation. Samples from the twelve fractions obtained by ultracentrifugation were run on a 12.5% SDS-PAGE gel to show the apoE distribution by immunoblotting. Arrow indicates the position of apoE.

3.2.6 Purification of apoE by ultra-filtration centrifugation

Purification of apoE was best achieved with 100 kDa MWCO ultra-filtration units from Amicon. These concentrators allowed us to achieve simultaneous concentration, purification and medium exchange. This has allowed us to attain a rapid and simple concentration of apoE particles from the conditioned medium. Concentrations of up to 100-fold were possible with minimal apoE loss. The large molecular weight cut-off limit on these units allowed for impurities to be removed, yet retarding the large apoE

particles. **Figures 3.10** and **3.11** show a Western blot and a complimentary Coomassie blue stain of concentrated and purified apoE3, apoE4, and apoE-free medium from native Neuro-2a cells, before (“*MEDIUM*”) and after (“*CONCENTRATED*”) purification. The contaminating higher molecular weight protein seen in the stain is likely albumin, which was present in the 10% FBS supplemented DMEM/F12 culture medium.

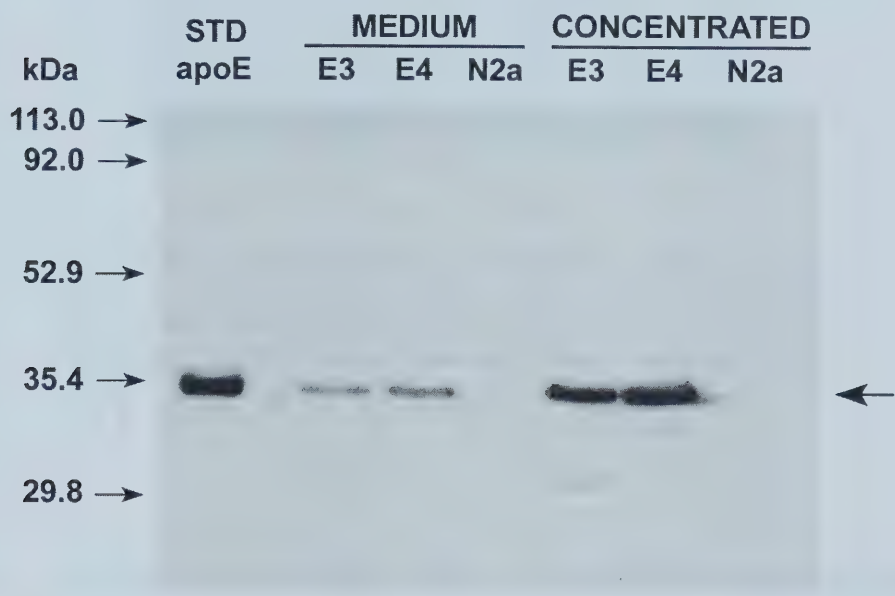


Figure 3.10 : Western blot analysis of samples purified by ultra-filtration. Amicon 100 kDa MWCO centrifugation devices proved to be the most effective method for purification and concentration of apoE lipid particles. Samples from cell culture medium and from the purified concentrated product were run on a 12.5% SDS PAGE. Samples obtained from commercially available apoE standard (“*STD apoE*”), secreted apoE3, apoE4, and native Neuro-2a cells were loaded in equal volumes per lane. Arrow indicates the position of apoE.

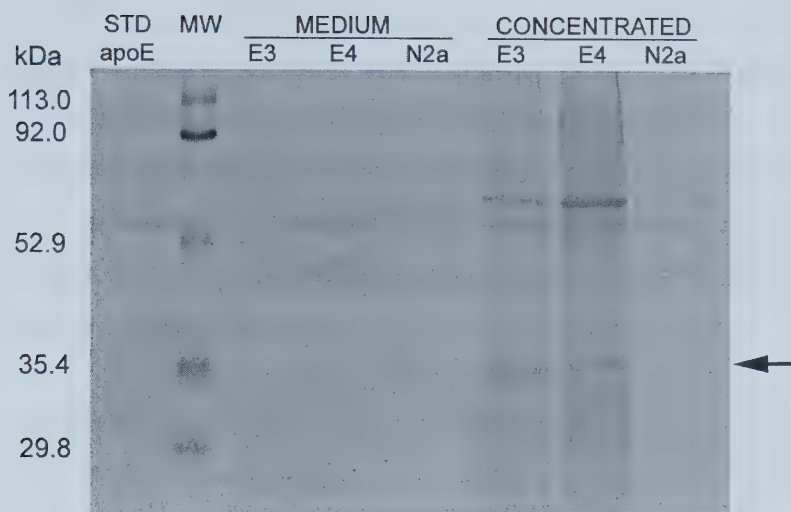


Figure 3.11 : Coomassie blue stain of apoE purified by ultra-filtration. Samples obtained from apoE3, apoE4, and native Neuro-2a cells were loaded in equal volumes per lane. “*STD apoE*”- commercially available apoE standard, “*MW*”- molecular weight marker. Arrow indicates the position of apoE.

3.3 EXPERIMENTS USING RAT SYMPATHETIC NEURONAL CULTURES

3.3.1 Preliminary Experiments

Previous studies in our laboratory (Rustaeus and Vance, unpublished) using partially purified N-terminal apoE fragments indicated that, in the presence of compactin, apoE3 stimulated and apoE4 inhibited the axonal extension of rat sympathetic neurons. However, these differences were somewhat ambiguous either due to the purity and low concentrations of apoE, or due to an overwhelming inhibition of cholesterol synthesis by compactin.

3.3.2 ApoE applied to neurons does not affect axonal elongation

To analyze the effects of lipidated human apoE3 and apoE4 particles on axonal elongation, we purified these lipid-associated proteins and applied them to rat sympathetic neurons grown in lipid-free medium in compartmented culture dishes.

Compartmented cultures of rat sympathetic neurons cells were grown for 7 days and axotomized as indicated previously in the **Methods** section. After axotomy, the neurons were incubated in the presence of delipidated rat serum and supplemented the medium with lipidated full-length apoE3, apoE4 or equivalent volume of apoE-free medium collected from native Neuro-2a cells. Axonal extension was measured every 24 hours. **Figure 3.12** shows that in the absence of cholesterol synthesis inhibition, neither full-length lipidated apoE3, nor apoE4, significantly affected axonal extension. This was in accord with our preliminary data obtained from the experiments using the N-terminal truncated forms of apoE (Rustaeus and Vance, unpublished). The cells grew as well as the control cells and in each condition the cells exhibited axonal extension in the range of $\pm 10\%$ of that of the control cells. The addition of apoE either to cell bodies/proximal axons only, or to distal axons only, did not cause any pronounced stimulatory or inhibitory effects on axonal elongation. As a result of this, further experiments were conducted under conditions of reduced cholesterol synthesis, in an attempt to induce apoE isoform-specific effects on axonal extension.

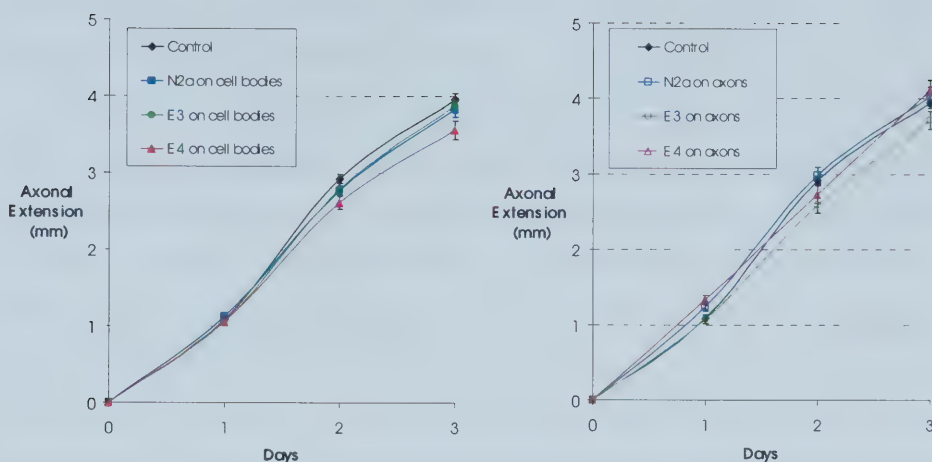


Figure 3.12 : ApoE3 and apoE4 treatment alone does not affect axonal extension. Sympathetic neurons were treated with 30 $\mu\text{g/ml}$ apoE3, apoE4 or supernatant from native Neuro-2a cells which do not secrete apoE ("N2a"). ApoE was added to either cell bodies/proximal axons alone (*left panel*) or to distal axons alone (*right panel*). Control cells were grown in medium with delipidated rat serum. Each condition was done in triplicate dishes with 32 tracks measured per dish ($n=96$). This experiment was completed twice and similar results were obtained. Error bars indicate standard error of the mean.

3.3.3 Dose-response of compactin inhibition of cholesterol synthesis using [^{14}C]acetate labelling

Previous results suggested that apoE affected axonal growth only when cholesterol synthesis was inhibited. Since compactin was to be used to inhibit cholesterol synthesis, a dose-response experiment was conducted in order to determine the effective concentration necessary for analyzing apoE isoform differences on axonal growth. As indicated in the **Methods** section, mass cultures of sympathetic neurons were treated with a range of compactin concentrations, from 0 to 1,000 nM, and incubated with delipidated rat serum in order to avoid any exogenous lipid interference from the serum. The cells were labelled with [^{14}C]acetate for 24 hours and

harvested. The incorporation of [^{14}C]acetate into [^{14}C]cholesterol therefore reflects the rate of cholesterol synthesis. The synthesized [^{14}C]cholesterol was isolated by TLC, and the radioactivity of each sample was counted. All samples had comparable total cell protein content. However, as compactin concentrations were increased, the cholesterol synthesis was progressively reduced. These assays allowed us to express the radioactivity in disintegrations per minute per μg of protein (DPM/ μg protein). As shown in **Figure 3.13**, a compactin concentration of 100 nM inhibits cholesterol synthesis by about 50%. Compactin concentrations greater than 500 nM have been shown to compromise cell viability and induce apoptosis (Michikawa and Yanagisawa 1998), and were not used in further experiments.

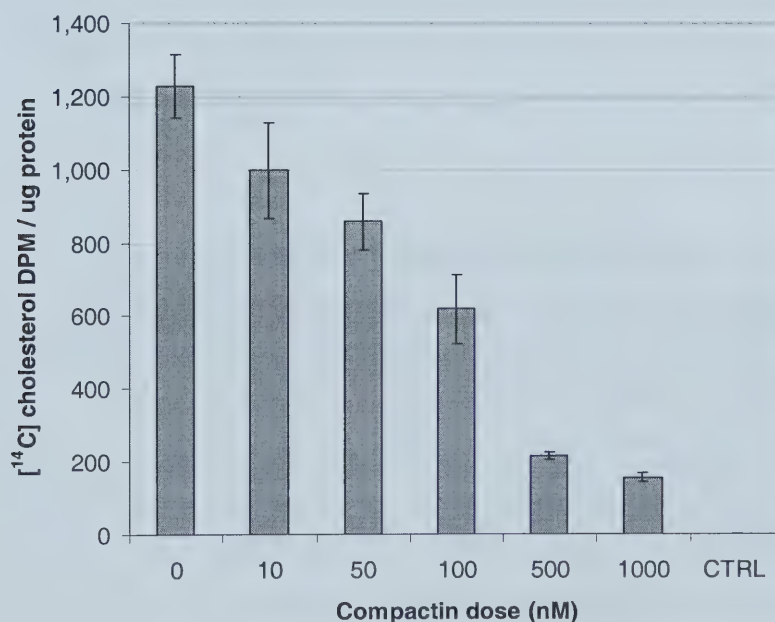


Figure 3.13 : Compactin dose-response inhibition of cholesterol synthesis in neuronal mass cultures. Cultures were labelled for 24 hours with [^{14}C]acetate, and incorporation into cholesterol was measured. “CTRL”- control cells incubated without radioactive label. Each condition was done in triplicate wells (n=3). The results displayed are from a single experiment. Error bars indicate standard deviation.

3.3.4 Compactin-induced inhibition of axonal extension

In order to determine how an inhibition of cholesterol synthesis affects axonal extension, compactin concentrations of 0, 50, 100, and 500 nM were applied to compartmented cultures of rat sympathetic neurons, and the extension of distal axons was measured.

Seven-day-old cultures were axotomized and incubated with a range of compactin concentrations in the presence of delipidated rat serum in the cell bodies/proximal axons compartment. **Figure 3.14** shows that when compactin was administered to cell bodies/proximal axons in increasing concentrations, the rate of axonal extension in the distal axon compartment was progressively reduced. Compactin had no effect on axonal extension when administered to the distal axons only, as previously demonstrated with another cholesterol synthesis inhibitor, pravastatin (Posse de Chaves et al. 1997).

To better analyze axonal growth, we harvested distal axons from these compactin-treated cultures, and performed a Western blot using anti-tubulin antibodies. Tubulin is a protein found in microtubules which form the cytoskeleton of axons. While the extension experiments measure the forward rate of extension of the fastest growing axon, immunoblotting against tubulin alternatively provides us with a direct measure of the overall axonal content. Upon visual inspection of these cultures, it was obvious that control cells treated with rat serum had thick cables of axons in the distal axon compartment, while the cultures treated with compactin in the presence of delipidated rat serum had much thinner and scarcer cables of axons. This was best portrayed by the Western blot analysis of tubulin content (**Figure 3.15**).

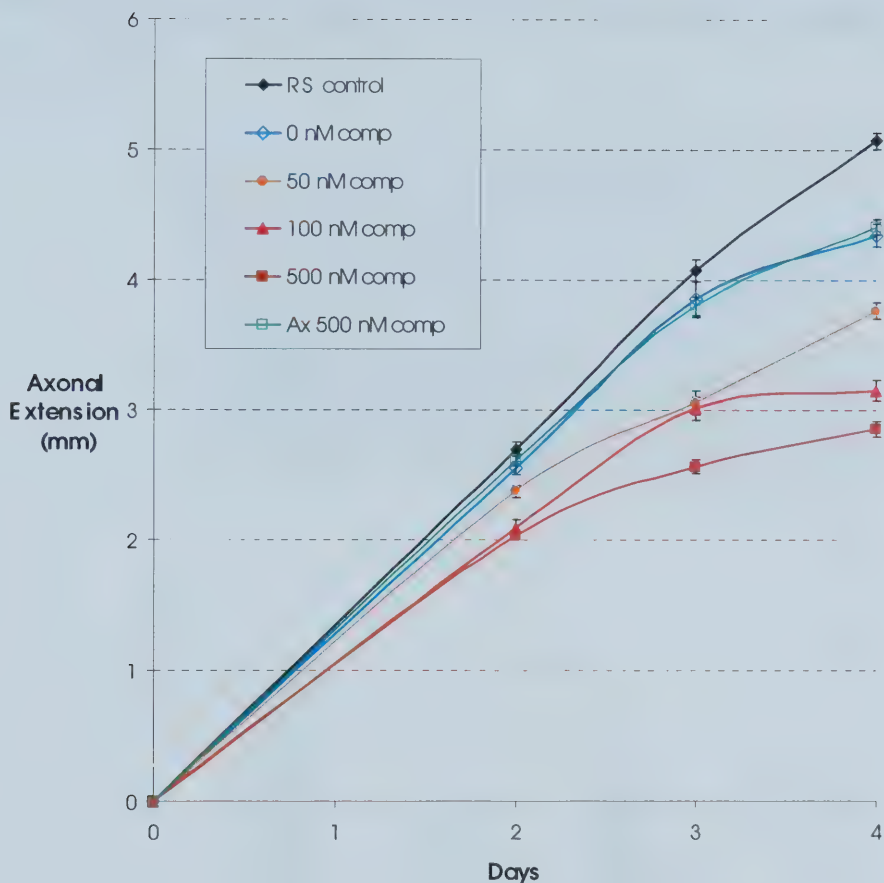


Figure 3.14 : Compactin inhibits axonal extension only when administered to cell bodies/proximal axons. All cultures were incubated with delipidated rat serum, except for the “RS control”, which had rat serum. Compactin was administered to cell bodies/proximal axons, except in the case of “Ax500” where it was added only to the distal axons compartment. All conditions were done in triplicate dishes with 32 measured tracks per dish (n=96). This experiment is a sample of two independent experiments with similar results. Error bars show standard error of the mean.

A comparison of **Figures 3.14** and **3.15** shows that while 50 nM and 100 nM compactin-treated neurons extend axons at a relatively similar rate, the tubulin content of the 100 nM compactin-treated neurons is greatly

reduced, indicating that under those unfavourable conditions, axons form thinner cables.

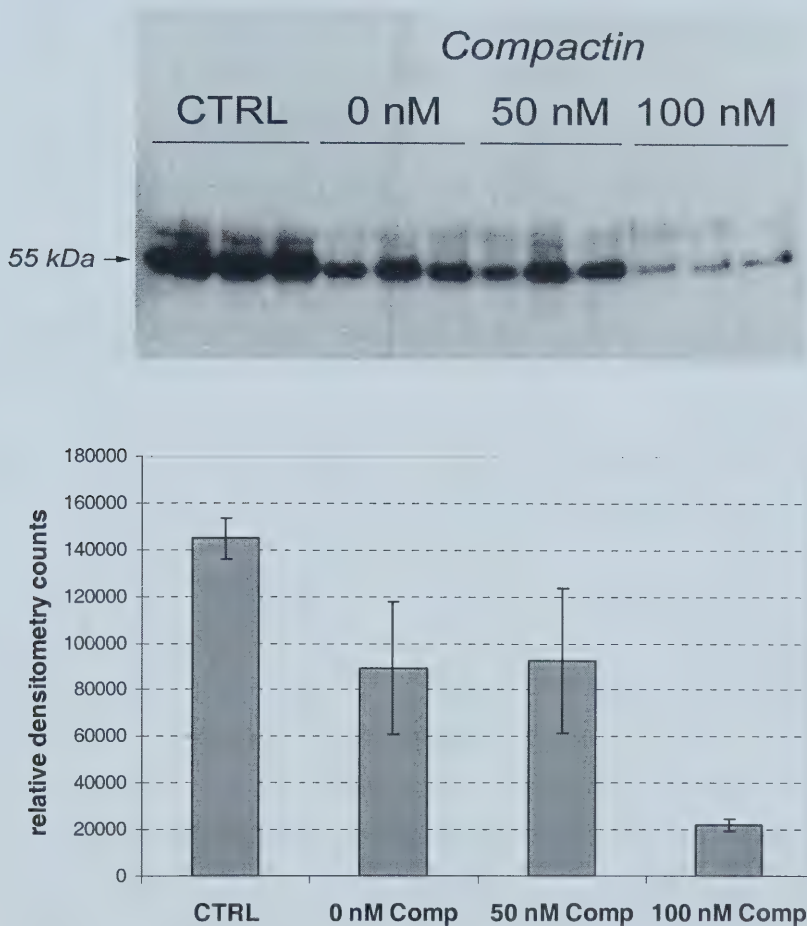


Figure 3.15 : Western blot analysis of tubulin content of distal axons of compactin-treated neurons. Axotomized neurons were grown for 5 days in the presence of delipidated rat serum and different compactin concentrations. Distal axons were harvested and equal sample volumes were run on an SDS-PAGE gel in triplicate to portray relative axonal tubulin amounts (*top*). The 55 kDa band corresponds to tubulin. “CTRL”- neurons grown in control medium with rat serum. The film results were quantified by densitometry measurements of tubulin bands (*bottom*). The data represent results from a single experiment. Error bars indicate standard deviation (n=3).

Pre-incubating cultures with compactin for 24 hours prior to axotomy was shown to enhance the inhibitory effect of compactin (**Figure 3.16**).

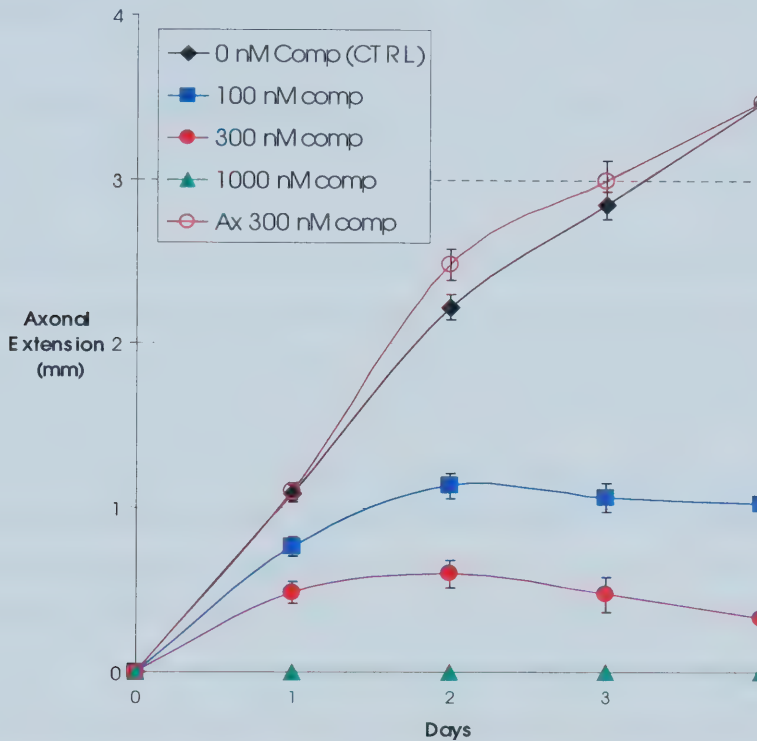


Figure 3.16 : High concentrations of compactin are detrimental to neuronal survival and axonal extension. Pre-incubating neurons with compactin for 24 hours prior to axotomy caused a pronounced inhibitory effect on extension, and doses higher than 100 nM compromised cell survival and caused neurons to retract axons. All cultures were grown in triplicate and treated with compactin in cell bodies/proximal axons compartment, while in “Ax300” the compactin was added to distal axons only. Error bars indicate standard error of the mean (n=96).

Since large concentrations of compactin tend to compromise the survival of neurons, and our goal was to experiment with healthy viable cells, we decided to use 50 nM of compactin in experiments involving apoE-treated neurons. At this concentration, the cholesterol synthesis was

inhibited by about 25% (**Figure 3.13**). However, with a one-hour pre-incubation of cells in this medium prior to axotomy, 50 nM compactin could induce 50% reduction of axonal extension without compromising survival (**Figure 3.17**).

3.3.5 ApoE has minimal effects on axonal extension in the presence of compactin

In experiments involving neurons treated with apoE under conditions of reduced cholesterol synthesis, we washed and pre-incubated the neurons with 50 nM compactin in lipid-free medium one hour prior to axotomy. This was done in order to ensure that all traces of rat serum-containing medium were washed off and lipids removed. Cells were axotomized and fresh lipid-free medium was added which included 50 nM compactin and 30µg/ml of apoE3 or apoE4, or equivalent volume of apoE-free medium obtained from native Neuro-2a cells. **Figure 3.17** shows that in comparison to the control lipid-free medium with 50 nM compactin, and apoE-free medium obtained from native Neuro-2a cells, both apoE3 and apoE4 supplemented media slightly enhanced axonal extension, however these effects were minimal. ApoE3 induced a 24% increase in extension while apoE4 induced a 15% increase. Most importantly, neither isoform exhibited any pronounced inhibitory effects on axonal extension.

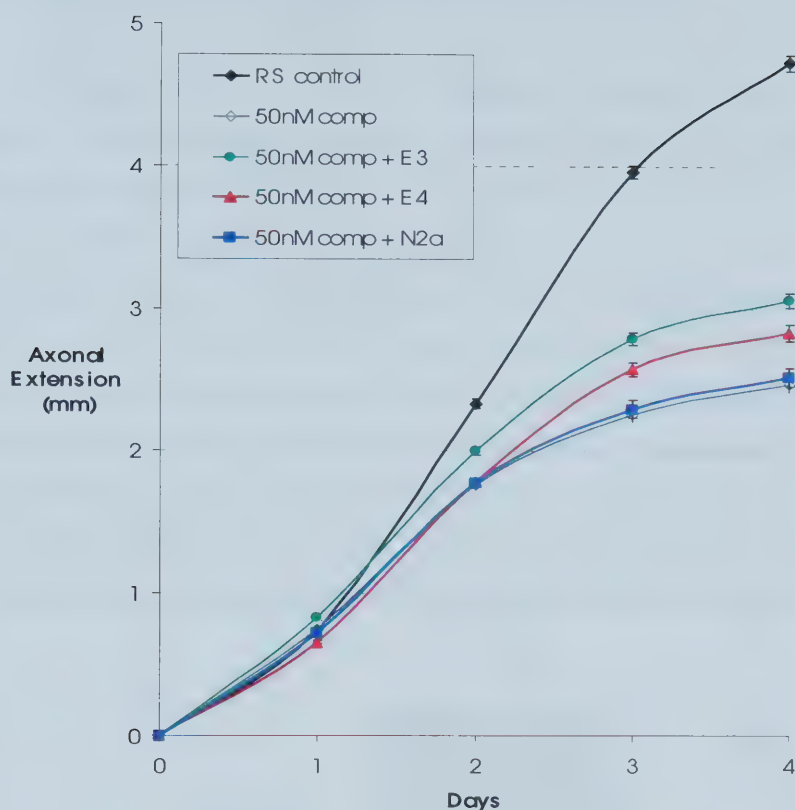


Figure 3.17 : ApoE4 does not inhibit axonal extension. One hour prior to axotomy, the neurons were incubated with medium containing 50 nM compactin and delipidated rat serum. Immediately after axotomy, 30 μ g/ml of apoE3, apoE4 and an equivalent volume of medium purified from native Neuro-2a cells were added to the cell bodies/proximal axons of neurons. All cultures were incubated in dRS and 50 nM compactin, except for “RS control” cells which were grown in the presence of rat serum. All conditions were done in triplicate dishes with 32 measured tracks per dish (n=96). Two other experiments yielded similar results, where apoE did not exhibit any significant isoform-specific effects on extension. Error bars indicate standard error of the mean.

3.4 GENOTYPING LDL RECEPTOR MUTANT MICE

In order for us to be able to determine whether LDL receptor is involved in apoE-mediated effects on neuron growth, we established a PCR-based genotyping system for LDL-receptor mutant mice (LDLR^{-/-}). This is a necessary step towards creating a breeding colony, and for genotyping animals for use in future experiments. As previously described, these mice lack a functional LDL receptor (Ishibashi et al. 1993). The mice were generated by disrupting the LDLR gene with the insertion of a *neo* cassette in the exon 4 of the LDL receptor gene. This disrupted mutant gene encodes a non-functional protein which is neither able to bind ligands nor associate with the cell membrane. **Figure 3.18** shows the two possible PCR products, an 800 bp mutant product and a 400 bp wild-type product.

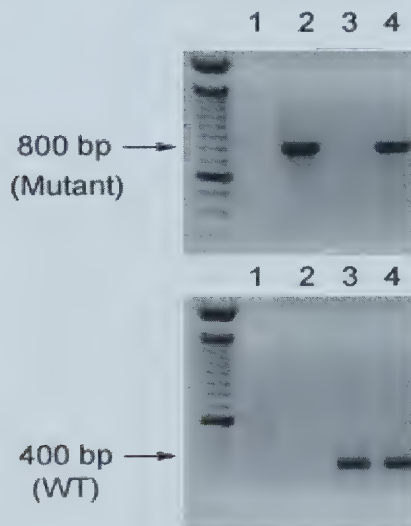


Figure 3.18 : LDL receptor mutant and wild-type PCR products.

The upper panel shows a reaction with primers for detecting a mutant allele, and the lower panel for detecting a wild-type “WT” allele. 1- Negative control, 2- LDLR^{-/-} mouse, 3- Wild-type mouse, 4- Heterozygous mouse. This 1.5% agarose gel was stained with ethidium bromide and exposed to ultraviolet light for visualization. The colors are inverted for printing purposes.

CHAPTER IV

Discussion

Our preliminary evidence suggested a link between the truncated N-terminal apoE4 fragment and the reduced ability of neurons to extend their axons in the presence of cholesterol synthesis inhibition. In those studies a relatively high (300 nM) concentration of compactin was used to inhibit cholesterol synthesis. Under these conditions the neurons had a tendency to retract axons. Since extensive inhibition of cholesterol synthesis may induce apoptosis in neurons, our goal was to reduce the concentration of compactin and analyze axonal extension in viable cells under conditions of reduced cholesterol synthesis and increased LDLR expression.

4.1 Discussion of apoE purification methods

Many studies have previously demonstrated that apoE3 has beneficial effects on neurons, while apoE4 tends to be detrimental. To further analyze these differences, we purified apoE isoforms in an attempt to use them in experiments with rat sympathetic neurons. Here we discuss some of the advantages and disadvantages of different purification methods, keeping in mind how the properties of apoE might influence the yield and success of the purification process.

At the beginning we attempted to isolate the N-terminal fragment of human apoE and analyze the purity of the apoE proteins that were used in the preliminary experiments. As indicated in the **Methods** section, the 22 kDa N-terminal apoE was expressed in bacterial cultures, concentrated by ultra-filtration centrifugation in 10 kDa filtration units and purified using heparin-Sepharose. The advantage of this model is that it can yield a large concentration of apoE (~10 mg/L) which was previously reported as >95% pure (Fisher et al. 1997). The disadvantage of this model is that it is not a full-length apoE protein, and it must be lipid associated with DMPC in order to be functional. Since a more physiological model would be to have full-

length apoE secreted on a lipid particle, we have opted to utilize stably transfected neuroblastoma cells which secrete minimally lipidated apoE particles. Purification of these particles proved to be a challenge due to the amphipathic properties of these particles. Using ELISA, we have determined that on average, these cells secrete about 5-10 μg of apoE / ml of medium.

At first, we used heparin-Sepharose affinity chromatography to purify these particles. Since heparin is a sulphated glycosaminoglycan and has a poly-anionic structure, it is capable of binding a multitude of proteins such as lipoproteins, lipases, hormone receptors, and proteins involved in DNA synthesis. The advantage of the heparin column is that apoE is known to interact with heparan sulphate proteoglycans on cell surfaces, and would naturally bind to the column. Unfortunately, since the column is not specific for apoE only, the purification process was compromised. Moreover, the purification process had to be followed by dialysis to remove salts, and certain amount of apoE would be lost in the process.

In order to enhance the purification process and increase the specificity for apoE, we used a monoclonal anti-apoE antibody column. This method improved the purity of apoE, however due to a small size of the column, the yield was limited. This method also required dialysis to remove the elution buffer, which further reduced the yield.

Since apoE protein is secreted on minimally lipidated particles, we used these features to our advantage. The particles have a reported diameter of 7-9 nm, and a density of 1.19-1.26 g/ml. As previously mentioned, these apoE3 and apoE4 particles have similar phospholipid and cholesterol composition, with one molecule of cholesterol per molecule of apoE, and no core lipids such as cholesteryl esters or triacylglycerols

(DeMattos et al. 2001). Based on the density and size, this places these particles in the range of poorly lipidated HDL particles (**HDL** 1.063-1.21 g/ml, 5-12nm; **LDL** 1.019-1.063g/ml, 18-25 nm; **IDL** 1.006-1.019g/ml, 25-35 nm; **VLDL** 0.95-1.006 g/ml, 30-80 nm; **chylomicrons** <0.95 g/ml, 75-1200nm) (Davis and Vance 1996). Because of this property, we were able to isolate the apoE particles using density gradient ultracentrifugation. However the disadvantage of this method is the low volumes used in the centrifugation and a resultant pure, but low yield. Extensive dialysis was similarly required to remove KBr salts from the product, as they are detrimental to neurons.

As a result, we have decided to use an ultra-filtration purification method using 100 kDa MWCO filtration units. Since the 34 kDa apoE protein is on a lipid particle which is much larger than the pores in the filter, this method allowed us to purify large quantities of medium and achieve yields of up to 500 µg/ml, which is more than 10-fold higher than the concentration required for experiments. Similarly, we were able to easily exchange medium from that used in the culturing of Neuro-2a cells to that which would be used in the neuron experiments.

4.2 Discussion of experiments involving rat sympathetic neurons

The experiments using rat sympathetic neurons have shown that lipid-associated human apoE particles exhibit no significant isoform specificity on the axonal elongation of rat neurons. This is in accord with our preliminary evidence using N-terminal apoE-DMPC associated particles. However unlike the preliminary experiments, cholesterol synthesis inhibition did not result in apoE4-mediated neurotoxic effects. In fact, both apoE3 and apoE4 particles slightly enhanced axonal extension in the presence of compactin, in comparison to control cells incubated without

apoE. At the end of a 4-day treatment, apoE3 enhanced the extension by 24%, and apoE4 enhanced it by 15%. These results imply that, under these culturing conditions, human apoE does not exhibit any significant isoform-specific effects on the axonal extension of rat sympathetic neurons. As a result, these experiments did not allow us to differentiate between the isoform differences with regards to lipid delivery or possible receptor-mediated signalling. Perhaps a different apoE concentration or a larger cholesterol inhibition might be required to induce isoform-specific responses.

Previously reported studies have used 5-30 µg/ml of apoE in experiments involving neurons and neuronal cultures. One study has shown that a concentration as low as 2.5 µg/ml of human recombinant apoE3 promotes the growth of mouse cortical neurons, while apoE4 inhibits growth (Nathan et al. 2002). While conditions for culturing peripheral nervous system neurons in compartmented cultures have been well established, the conditions for culturing central nervous system neurons are currently under investigation. We have therefore used a currently available model for culturing peripheral neurons. Since neurons used in this study are obtained from cervical sympathetic ganglia, and are part of the peripheral nervous system, the concentration of apoE applied to these neurons had to closely resemble the concentration of apoE found in the plasma. Similar to other studies, we have therefore used 30 µg/ml of apoE in the experiments. Recent studies in our lab have shown that in rat sympathetic neurons, the LDL receptor is present mostly in the cell bodies/proximal axons and very little in the distal axons (Posse de Chaves et al. 2000). Furthermore, treatment with inhibitors of cholesterol synthesis (pravastatin) increased the expression of the LDL receptor in the cell bodies/proximal axons of sympathetic neurons. Hence, we administered apoE only to the cell bodies/proximal axons of neurons. Compactin was

administered to cell bodies/proximal axons only, since it was previously shown that, unlike phospholipids, cholesterol is synthesized only in cell bodies/proximal axons (Vance et al. 1994). This was demonstrated by the experiments showing that addition of pravastatin (an inhibitor similar to compactin) to cell bodies/proximal axons inhibited cholesterol synthesis and axonal extension, and that re-supplying cholesterol rescued growth (Posse de Chaves et al. 1997). Addition of these statins to distal axons did not inhibit axonal elongation, suggesting that the effects of statins are local, and any possible retrograde transport of statins is insufficient to inhibit cholesterol synthesis enough to affect axonal elongation.

In addition, previous studies from our laboratory showed that the LRP receptor was present in the CNS of rats, however, LRP receptor could not be detected in rat sympathetic neurons, which would imply that the LDLR might be the primary receptor involved in the apoE binding (Posse de Chaves et al. 2000). In light of this evidence, LDLR $-/-$ mice could prove to be important in determining which of these receptors is responsible for apoE binding, and whether the LRP receptor is responsible for isoform-specific differences of apoE.

4.3 Future directions

Our goal was to investigate apoE isoform-specific effects under conditions of reduced cholesterol synthesis, where LDLR up-regulation might be responsible for enhancing the signalling effects of apoE isoforms. Possibly, a higher compactin concentration might be necessary to observe apoE isoform-specific effects. Perhaps with higher compactin concentrations, the culturing conditions might be unfavourable for survival and resemble those conditions found in the diseased brain, and perhaps the neurons might have to be borderline viable in order for apoE to exhibit

isoform-specific effects. Since we could not confirm that addition of apoE4, but not E3, to cell bodies/proximal axons inhibits axonal extension when cholesterol synthesis is inhibited, further studies must be done to clarify this phenomenon.

The focus of current reviews has been on elucidating the role of cholesterol in the development of Alzheimer's disease (Yanagisawa 2002). In fact, both hypercholesterolemia and atherosclerosis have been linked to the incidence of dementia (Hofman et al. 1997). If most of cholesterol required by the brain is derived in situ, how does high cholesterol in plasma affect CNS neurons which are protected by the blood-brain-barrier and predispose them to Alzheimer's disease? The fact that apoE expression is up-regulated during injury implicates apoE in recycling of lipids at the site of damage (Seitz et al. 2003). However lipid delivery might not be the only beneficial effect of apoE. Possible receptor-mediated signalling roles of apoE involving adapter proteins Dab-1 and FE65 have been postulated since they have been found to interact with LDL and LRP receptors, as well as cellular tyrosine kinases which can generate intracellular signals (Trommsdorff et al. 1998). Other protein kinases such as **c-Jun N-terminal kinase (JNK)** and **mitogen activated protein Kinase (MAPK)** have also been implicated in signalling through LDL receptor family members (Gotthardt et al. 2000). Other studies have focused on pathways involving **protein kinase B (PKB)** and **phosphatidylinositol 3-kinase (PI3K)** (Laffont et al. 2002), as well as **protein kinase A (PKA)** (Ohkubo et al. 2001). These kinases may be involved in cytoskeletal reorganization by altering the phosphorylation states of cytoskeletal proteins such as tau, whose aggregation may accelerate the progression of the disease.

Future studies should focus on the role of cholesterol in apoE function, and the interaction of apoE with the LDL receptors. Since this

study was unable to measure any significant isoform-specific effects of apoE on neurons, further studies should be conducted to examine which conditions would allow apoE4 isoform to inhibit axonal extension. It should also be determined whether exogenously administered cholesterol can rescue the growth. Since LDLR is found primarily in the cell bodies/proximal axons, addition of apoE to distal axons only would allow us to determine if LDLR or perhaps some other receptor is responsible for the effects on extension. Perhaps we could experiment with lipid particles such as HDL, whose lipid content is larger than the minimally lipidated apoE particles which were used in this study. Perhaps there is not enough lipid present in the particles to induce apoE isoform-specific effects. We should also determine why the inhibition of cholesterol synthesis is required to induce apoE effects on nerve growth. Perhaps lipid delivery is important, and perhaps lipid modification of apoE to make a functional signalling molecule bears even more importance. However, these two suggestions may not be mutually exclusive and may both be involved in a mechanism of apoE isoform-specific effects. Perhaps the effects of apoE isoforms could be analyzed by over-expressing the LDLR in neurons without inhibiting the cholesterol synthesis. Furthermore, if there is a difference in apoE isoform binding to the LDLR and subsequent intracellular signalling, then this difference would be eliminated in the LDLR^{-/-} mice. Most importantly, since Alzheimer's disease is a disease of the CNS, we should apply these principles to CNS neurons, such as hippocampal preparations or preparations of retinal ganglion cells, in order to make our models more physiologically relevant.

4.4 Concluding remarks

Since Alzheimer's disease is a progressive neurodegenerative disorder that requires many years to develop, it is very difficult to assess which small changes in the body's metabolism can accumulate over time to create a cumulative neurotoxic effect. The complex nature of lipid metabolism intertwined with the unknown function of β -amyloid protein creates a multidimensional disease influenced by a multitude of factors which may puzzle the minds of researchers for years to come. Until these issues are resolved, the role of apoE in the Alzheimer's disease will remain a mystery.

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